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Quantitative Studies on the Multiplication of Neoplastic Cells *in Vivo*. I. Growth Curves of the Ehrlich and MC1M Ascites Tumors [1, 2]

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It has been established in recent years that ascites tumors have many advantages over solid tumors for use in several types of investigations in various branches of experimental oncology. Ascites tumors have been used for cytological, biochemical, immunogenetical, and other studies of neoplastic cells and were also useful in experiments concerning growth-inhibition [(*1–11*) and others]. A number of different tumors can be induced to grow in the ascites form (*1*, *3*, *5–7*) which, according to previous definitions (*12*, *5*), means a condition where free tumor cells or cell complexes grow actively in the peritoneal fluid, induce ascites accumulation, and reach there a high absolute and relative concentration.

The problem of quantitative measurement of ascites tumor growth has not been adequately solved. A number of indirect criteria, such as the increase in weight of the host animals (*2*), survival time (*2*, *13*, *6*), and changes in the cellular composition of peritoneal fluid (*7*) have previously been used to judge the growth rate of some ascites tumors. No attempts

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[2] This work has been made possible by grants from the Wallenberg Foundation, Kvinnogillesförbundet, the Swedish Cancer Society, and Lotten Bohman's Fund.

[3] With the assistance of Elin Klein. Our sincere thanks are due to Prof. T. Caspersson and Dr. Eva Klein for stimulating criticism, to Mrs. Ulla Lomakka and Mr. L. Aquilonius for valuable technical assistance, Dr. T. S. Hauschka for supplying us with breeder pairs of the inbred mouse strains, and Dr. E. E. Miller for the MC1M tumor.

have been made to measure directly the increase of the *total* number of free tumor cells in the entire peritoneal fluid and thus obtain quantitative growth curves. The obtaining of such direct growth curves appeared to be of interest, for the following two reasons: In studies on biochemical or other distinctive features of neoplastic cells, an exact and detailed knowledge of the rate of cell multiplication at any given time would often be necessary. In the course of such investigations the question often arises as to whether detected differences between homologous normal and neoplastic tissues are being caused by the neoplastic process, or by the rapid growth of the tumor *per se*; comparison with the homologous embryonic or regenerating tissue having the same growth rate as the tumor studied is then necessary. Secondly, the course of tumor-cell multiplication *in vivo* is still quite unknown. The only available data are those obtained by measuring the volume, weight, diameter, etc. of solid neoplasms, and these studies do not permit any direct inferences about the fundamental growth characteristics of the neoplastic cells.

In the present investigation, the authors have attempted to determine quantitatively the changes in the total number of free tumor cells within the entire peritoneal fluid of the mouse during the whole period of ascites tumor growth. Data have also been secured on the viability of the tumor-cell population at different times, the average cellular volume, the mitotic index, the average length of the intermitotic period and of mitosis, and the progression of tumor infiltration into various tissues of the peritoneal cavity. Among the possible factors that might influence the shape of the growth curves, the size and the physiological age of the inoculum were studied particularly. Detailed investigations were made of the changes in the number of non-tumor cells of the host being intermixed with the tumor cells at different times after inoculation, since the relative proportion of these cells determines how closely the ascites tumor can approach the condition of a "pure culture." Finally, the relationships between the increase in number of ascites tumor cells and the progressive accumulation of peritoneal fluid were studied.

Two ascites tumors were used, the Ehrlich ascites carcinoma (*1, 2, 4, 5*) and the MC1M ascites sarcoma (*5*). The growth curves obtained were compared with data on the growth characteristics of solid tumors, of rodent embryos, and of populations of unicellular organisms.

Materials and Methods

Mice

C3H/St mice, received from Dr. T. S. Hauschka in 1950 and kept by brother × sister matings through 7 generations in our laboratory, and F_1 hybrids produced by mating C3H/St mice with mice from 4 different inbred strains (A/St, C57BL, C57L, and DBA[4]) were used for most of the experiments. Some preliminary studies on the Ehrlich ascites tumor have been carried out with heterozygous albino mice. The animals used were

[4] "Behaves immunologically like DBA/2"—Hauschka, T. S.: Mouse News Letter # 8.

of both sexes, 2 to 4 months old, and weighed 18 to 25 grams. They were given a standard compressed diet and drinking water *ad libitum*.

Tumors

a) The Ehrlich ascites tumor was produced from a solid Ehrlich carcinoma (received from Dr. Albert Fischer, Carlsberg Foundation, Copenhagen, Denmark) in 1948, and has since been kept by serial ascitic fluid transfers (over 200 passages) in hybrid mice. It was found to grow progressively in 100 percent of the hybrid albino, C3H/St, A/St, DBA, C57BL, and C57L animals, and in F_1 hybrids produced by any combination of these 5 inbred strains. Routinely, 0.1 to 0.2 ml. of the undiluted ascitic fluid was transferred intraperitoneally. With this technique, no spontaneous regressions were observed in more than 2,000 mice. Stained smear preparations of the Ehrlich ascites tumor are shown in figures 1 and 2.

b) The MC1M ascites tumor was produced in 1950 from a solid transplantable sarcoma originally induced in 1945 by methylcholanthrene in C3H mice, by Miller *et al.* (*14*). Data on this tumor and its gradual transformation into an ascites tumor are to be found in a previous paper (*5*). When used for the present experiments, it had passed through more than 80 intraperitoneal ascitic-fluid transfer generations. It was found to grow progressively in nearly 100 percent of C3H mice and F_1 hybrids from C3H and any other strain. Stained smear preparations of the ascitic fluid are shown in figures 3 and 4. A line of the original solid tumor, maintained by subcutaneous transplantation, was also used for some of the experiments. This line has never been transplanted to the peritoneal cavity.

Procedure

Two series of experiments were carried out with the Ehrlich ascites tumor. In Series I the physiological age of the inoculum was kept constant (6 days), while the inoculum dose was varied between 0.7 and 40 million tumor cells. In Series II the inoculum dose was kept constant (1.8×10^6 tumor cells) while the age of the inoculum was varied between 3 and 16 days.

Ascites was collected from mice used in the routine transplantation of the Ehrlich ascites tumor. Mice showing marked abdominal distention were selected at random. Their ascitic fluid was combined and about 10 million cells from the pooled fluid were injected into a group of 3 to 10 mice. In Series I, these animals were killed and their peritoneal fluid collected after 6 days. In Series II, the same was done after a predetermined number of days for each experiment (3, 6, or 16 days respectively). One aliquot of ascites fluid was taken for the determination of the total number of cells per ml., while another part was used to prepare wet smears that were fixed and stained according to the method of Papanicolaou. The total number of cells per ml. was established by counting in a Buerker hemocytometer after tenfold dilution of the fluid with saline, followed by another tenfold dilution with Türck's solution. The percentage of tumor cells was determined from the stained smears [for details of the differential

counting see (*5*)]. While these procedures were carried out, the main bulk of the fluid was stored in the refrigerator at 2 to 4° C. for 2 to 6 hours. The number of tumor cells per ml. of ascites was computed, dilutions were made with 0.9 percent NaCl solution, and requisite numbers of cells ($0.7–40 \times 10^6$ in Series I; 1.8×10^6 in Series II) were inoculated with an Agla micrometer syringe into 40 to 50 mice in every experiment. The injections were completed within 1 hour after the dilution was made. In making the dilutions, care was taken to keep the volume of the inoculum between 0.2 and 0.4 ml. in the different experiments.

Pairs of mice were killed at regular intervals after the inoculation for as long as animal survival permitted. The mice were sacrificed by dislocation of the neck, the abdominal skin was removed, and a small opening was cut in the abdominal wall. Available ascites was collected with a syringe. One drop of a 20 percent potassium oxalate solution was added to avoid possible clotting. The peritoneal cavity was then rinsed with 1-ml. lots of saline. The saline was injected through the incision, moved around in the abdominal cavity by gently moving the mouse, and aspirated with the same syringe used for the injection. The lots of fluid from each mouse were pooled, using great care to avoid any loss of fluid. The rinsing was carried out repeatedly until the saline appeared quite clear when withdrawn from the peritoneal cavity—generally after 10 to 50 ml. of saline per mouse had been used. The final rinsing fluid usually appeared cell-free upon microscopic examination and only occasionally could a few cells be detected in it.

The volume of the pooled liquid, containing all ascites and rinsing fluid collected from one mouse, was then measured. One aliquot of the material was taken for cell counts and the total number of nucleated cells was determined in the Buerker chamber after dilution with Türck's solution. The rest of the fluid was centrifuged, the sediment was resuspended in small amounts of serum, and a smear was prepared according to the technique of Papanicolaou. Tumor cells and non-tumor cells on the smear were differentially counted. When the total number of nucleated cells and the percentage of tumor cells were known, the total number of tumor cells and of non-tumor cells in the entire peritoneal fluid of one mouse could be calculated.[5]

The mitotic index of the tumor cells was determined in several experiments. Two thousand cells were counted in each smear preparation. More detailed data on the mitotic counts will be given below, in connection with the errors of the methods.

Tissue samples from different regions of the peritoneal cavity (mesenterium, omentum, fatty tissue, pancreas, parietal and visceral peritoneum, etc.) were collected, fixed with 10 percent formalin, embedded in paraffin, sectioned at 5 μ, and stained with hematoxylin and eosin. The sections were examined to determine the time at which implantation of tumor cells starts under the conditions of the different experiments in the various tissues examined.

[5] We wish to express our thanks to Dr. J. L. Ambrus, College of Pharmacy and Science, Philadelphia, Pa., who suggested the use of the rinsing technique.

Average cell volume was estimated at varying intervals after inoculation by the following procedure: Simultaneously with the determination of the total number of ascites tumor cells per mouse, 1 to 5 additional mice (depending on the quantity of peritoneal fluid available) were punctured with a capillary glass pipette and the readily available peritoneal fluid was collected and pooled. The number of cells per ml. was counted and the percentage of tumor cells estimated as described above. Samples of the fluid were centrifuged in a hematocrit tube until the volume of corpuscular sediment remained constant (about 30 minutes at 3,000 $\times$ g). With the ratio of the volumes of sediment and supernatant and the number of cells per ml. known, the volume of the average cell could be calculated. As both Ehrlich and MC1M ascites tumor cells are larger than most non-tumor cells occurring in the peritoneal exudate (*5*), an increasing admixture of non-tumor cells in the exudate will tend to lower the average cell volume. For this reason only values from cell populations containing more than 80 percent of tumor cells were taken into consideration.

The viable condition of the cells was judged according to the method of Schrek (*15*). Fresh ascitic fluids were used, obtained by puncturing the mice in the same way as described above for the cell-volume determinations. The fluids were diluted 1:20 with Tyrode's solution, containing 0.05 percent eosin. After gently shaking the mixture for two minutes, a drop was put into the chamber of a hemocytometer. The number of stained and unstained cells was then quickly counted.

Experiments with the MC1M ascites sarcoma (Series III) were carried out with the same procedures used for the Ehrlich ascites tumor. For the MC1M sarcoma the effect of inoculum age on the growth curves was not investigated.

Errors of the Methods

I. Errors of Cell Counting

Two dilutions were made from each sample when the concentration of cells per ml. was to be established. Four squares were counted in one chamber for each dilution. The standard error of the mean could be calculated from the differences between the duplicate dilutions, and was found to be 7.1 percent. This is consistent with the error found in previous experiments of Goldberg, Klein, and Klein (*13*), where the standard error was 6.8 percent with essentially the same procedure.

II. Errors of Differential Counting of Tumor Cells and Determination of Mitotic Index

Errors were established from duplicate counts performed on 50 different smear preparations. Two thousand cells were counted on each slide. The cells were classified as tumor cells [*T*-cells—for cytological criteria see (*5*)] in interphase, tumor cells in mitosis, and non-tumor cells (*n*-cells). Mitotic figures of all stages from well recognizable prophase to the separation of the daughter cells were counted. The percentage of *T*-cells and of *n*-cells, and the mitotic index (*i.e.*, the number of *T*-cells in mitosis per 100 *T*-cells) were calculated for each 1,000 cells counted. The difference in *T*-cell and *n*-cell percentages and in mitotic indexes between the first and second thousand cells counted on the same slide were then computed for the whole material. The standard deviation of these differences (σ_d), divided by $\sqrt{2}$, is the standard error of a single count-

ing (σ_s) performed on 1,000 cells (*16*). As the mitotic indexes were based on 2,000 cells, the standard error of the mean for the two duplicates, each based on 1,000 cells, was calculated in their case $\left(\sigma_M = \frac{\sigma_s}{\sqrt{2}}\right)$. The results are given in table 1.

TABLE 1.—*Errors of differential counting and of mitotic indexes*

	Number of duplicate counts	Mean	Standard error of a single determination	
			σ_s	Percent of mean
T-cells in percent	50	76.51	2.11*	2.75*
n-cells in percent	50	23.49	2.11*	8.98*
			σ_M	
Mitotic index	49	2.79	0.43†	15.32†

*When counting 1,000 cells.
†When counting 2,000 cells.

III. Variability in the Determination of the Total Number of Tumor Cells in the Entire Peritoneal Fluid

As pairs of mice were killed at each time interval after inoculation, the difference in total number of tumor cells found in the peritoneal fluid of each of the two animals could be used to estimate the variability involved in these determinations. This variability is a composite one. It is partly due to the variability among animals of the total number of free tumor cells in the peritoneal fluid at the same time after inoculation with the same number of tumor cells from the same pool. Furthermore, the errors of all procedures used in determining the total number of tumor cells are involved; for example, in the rinsing procedure, errors of volume measurements of the combined rinsing fluids, determination of cell number per ml., and tumor-cell differential counting. Not all of these different errors are open to an experimental determination. The final composite variability, determined from the difference between parallel determinations on two different animals, is, however, quite sufficient for the present purpose, as it gives an estimate of the reliability of the experimental values.

The logarithms of the numbers of tumor cells were used for the calculations because they, and not the numbers themselves, were found to be approximately normally distributed.

The data obtained in the experiments of Series I and II for the total number of tumor cells per mouse were divided into four groups, with logarithmic values between 5–7, 7–8, 8–9, and 9–9.5, respectively. The standard deviation of the differences (σ_d) between the duplicate values obtained from two different animals at the same time was calculated for each group. The error of a single determination, σ_s, is obtained after dividing σ_d by $\sqrt{2}$ (*16*). The standard error of the mean between two parallel determinations, σ_M, can be calculated by dividing σ_s by $\sqrt{2}$ or σ_d by 2. Both σ_s and σ_M are obtained in logarithmic units. To transform the log units into percentages of the mean the following ordinary formulas were used:

$$+\sigma \text{ percent} = 100 \times \text{antilog } \sigma - 100$$

$$-\sigma \text{ percent} = 100 - 100 \times \text{antilog } (-\sigma)$$

The results obtained from 72 duplicate counts are given in table 2. It appears that the error decreased considerably with increasing numbers of tumor cells. This indi-

TABLE 2.—*Variability in the determination of the total number of tumor cells in the entire peritoneal fluid of the mouse, as established from differences between duplicate determinations*

Group	Number of tumor cells	Number of duplicate counts	Mean of the logarithms of tumor-cell numbers	Error of a single determination			Error of the mean between duplicate determinations		
				in log units σ_s	as percent of the mean		in log units σ_M	as percent of the mean cell number	
					$+\sigma_s$ percent	$-\sigma_s$ percent		$+\sigma_M$ percent	$-\sigma_M$ percent
1	2	3	4	5	6	7	8	9	10
1	10^6–10^7	10	6. 0088	0. 2270	68. 65	40. 71	0. 1605	44. 7	30. 9
2	10^7–10^8	28	7. 5540	0. 1440	39. 30	28. 23	0. 1015	26. 3	20. 8
3	10^8–10^9	25	8. 4350	0. 1130	29. 60	22. 91	0. 0800	20. 2	16. 8
4	10^9–$10^{9.5}$	9	9. 1047	0. 0629	15. 60	13. 49	0. 0445	10. 8	9. 7
	Average		7. 8940	0. 1410	39. 47	27. 19	0. 0996	26. 3	20. 2

cates that the percentage error is not homogeneous or identical for all values within any of the four classes. The error is underestimated for values lower than the group mean, and for higher values it is exaggerated. A further subdivision of the data into smaller groups would minimize this difference within the groups. The size of the material did not make such a subdivision practical, however, nor was it considered necessary for the purpose of the present study. The values of σ_s, listed in table 2, were useful when drawing curves through the experimental points, giving an estimate of their reliability. The limits of $\pm\sigma_s$ were indicated graphically around the mean of each pair of determinations (*cf.* text-figs. 1, 9, and 11), and they are interpreted to mean that, for similar sets of data, the values of logarithmic tumor-cell numbers could be expected two times out of three to differ from our data by not more than $\pm\sigma_s$. Strictly, this is true only for values in the neighborhood of the mean of each group, but it represents a reasonable approximation for all the data.

The average error of a single determination is much larger (33.3 percent) than the error of cell counting (7.1 percent) or that of differential counting (2.75 percent). It must be concluded that the main variability involved in the determinations of the total number of tumor cells in different individual animals is due to other factors. These are primarily the errors of the rinsing procedure and the variability among animals with respect to the number of free tumor cells present in the peritoneal fluid at the same point of time. The latter depends partly on variations in inoculum dose (the error of the inoculation) and partly on variations in the growth history of the cells in different mice.

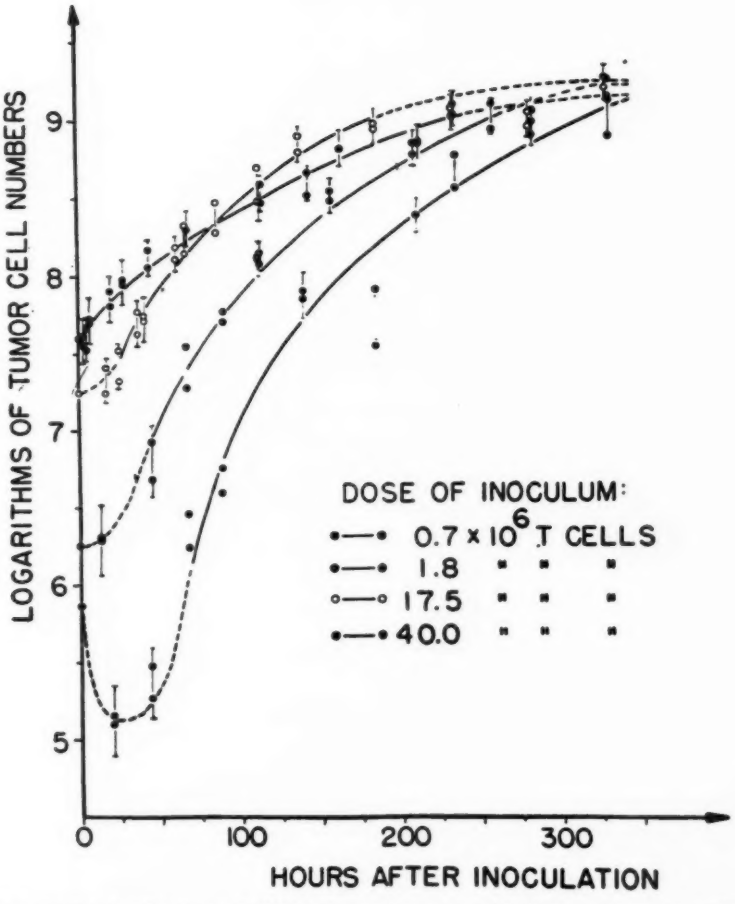
Results—Series I

A. The Course of Tumor-Cell Multiplication

The experiments of Series I were performed with the Ehrlich ascites tumor by varying the dose of inoculum and keeping its physiological age constant (6 days). The lowest inoculum used was 0.7 million tumor cells, since this is in the neighborhood of the critical dose necessary to produce ascites consistently in 100 percent of the animals (*6*).

Text-figure 1 shows the growth curves obtained. It appears that the total number of tumor cells remained constant or decreased for a short period after inoculation. This period was longer with the lower doses of inoculum than with the higher ones, and when 40 million cells were inoculated it was not detectable at all. The tumor cells started to increase in number rapidly thereafter. This increase was never truly exponential (the logarithms of tumor-cell numbers did not fall on a straight line). All four curves reached a final maximum value of the same order of magnitude (1,500–2,000 million tumor cells). There was no increase beyond this range of cell numbers and the host animals died.

It has often been assumed that multiplication of tumor cells proceeds exponentially *in vivo*, at least for a period of time (*17–20*). The complete absence of a true exponential phase during ascites tumor growth was therefore quite surprising at first. Studying available data on solid tumor growth it appeared, however, that Mayneord (*20*), Schrek (*21–24*), and others have found that the diameter of certain solid tumors increases linearly with time. A linear increase of diameters corresponds to a cubic and not to an exponential increase of the volumes. It became of interest to compare ascites and solid tumor growth curves to ascertain whether they are basically similar or not.



TEXT-FIGURE 1.—Increase of the total number of free Ehrlich ascites tumor cells in the entire peritoneal fluid of the mouse. Four different cell doses with the same physiological age (6 days) were used for inoculation (Series I). Each point was obtained by using a different mouse. The standard error of each single determination is indicated. The dotted lines are freehand drawings, while the solid black lines represent equation (1) fitted to the data.

As the average volume of the individual ascites tumor cell does not change significantly during the major part of the growth period (see below), the total number of tumor cells is simply proportional to their total volume, and is thus a measure comparable to the volume of an "ideal" solid tumor, composed mainly of living tumor parenchyma. If the same law would describe the growth of our ascites tumors and Schrek's (*21–24*) "linear" solid tumors, it could be expected that the cube roots of the numbers of ascites tumor cells would fall on a straight line when plotted against time, as the cube roots are then comparable to the diameters of solid tumors.

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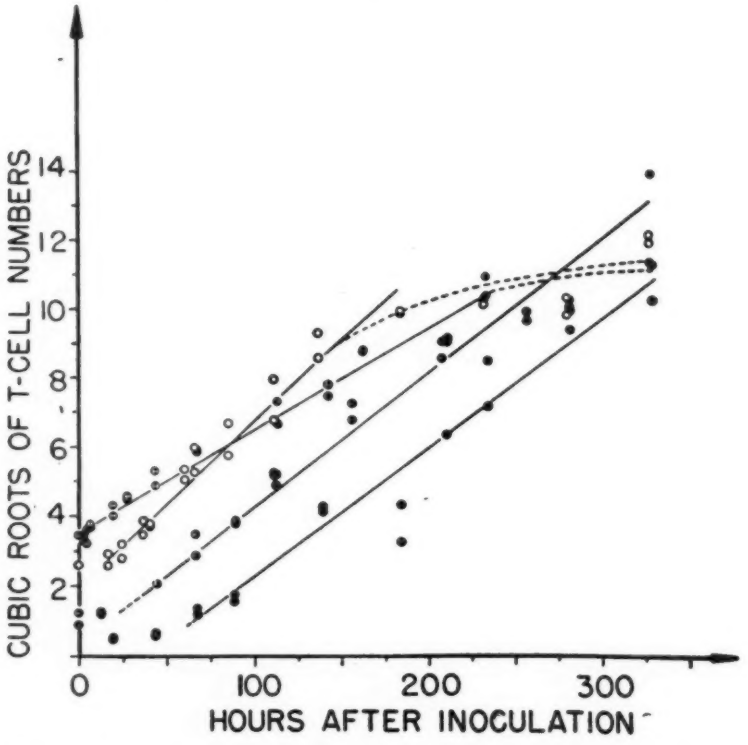
In order to test this possibility the cube roots of tumor-cell numbers (total number of free tumor cells per mouse) were plotted against time. With the exception of the earliest and latest stages of growth, the points were indeed found to fall within the reasonable proximity of a straight line (text-fig. 2). The regression data given in table 3, column 4, indicate the time intervals included in the calculations.



TEXT-FIGURE 2.—The same as text-figure 1, except that the ordinate now represents the cube roots of tumor-cell numbers ($\times 10^{-2}$) instead of the logarithms. The solid black lines are the calculated regression lines; the dotted lines are freehand drawings.

As appears from columns 7 and 8 of table 3, the departure from the regression line decreased with increasing doses of inoculum. This is at least partly due to the fact that the experimental error decreases with increasing cell numbers (*cf.* table 2).

The satisfactory fitting of the straight regression lines to the experimental data seems to justify the description of the curve parts in question (*i.e.* the parts within the time intervals given in column 4, table 3) by the equation of the lines written in the following form:

$$\sqrt[3]{N}=b(t-t_o)+\sqrt[3]{N_o} \qquad (1)$$

TABLE 3.—*Regression data for the straight-line relationship between the cube roots of ascites tumor-cell numbers and the time (in hours) after inoculation*

Dose of inoculum (*number of tumor cells*)	Age of inoculum (*days*)	Terminal point of growth curve (*hours after inoculation*)	Time interval for straight regression line (*hours after inoculation*)	Regression (*cube roots of tumor-cell numbers/time*)				Standard deviation of the differences between duplicates (σ_d) (*Same units as in column 7*)	t_o (*hours*)
				No. of points	Regression coefficient $b \pm \sigma_b$	Departure from regression line (σ_{y-Y})			
						Cube roots of *T*-cell numbers	Percent of mean ($\bar{y}$)		
1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Series I:									
0.73×10^6	6	331	68–331	19	3.75 ± 0.34 (*a*)	128	23.0	93	63.8
1.80×10^6	6	330	45–330	20	3.91 ± 0.21 (*b*)	72	12.3	57	22.8
17.50×10^6	6	329	17–183	24	4.78 ± 0.24 (*c*)	49	9.2	53	13.6
40.00×10^6	6	331	1–234	30	2.98 ± 0.09 (*d*)	37	6.4	38	(−2.6)
Series II:									
1.80×10^6	3	229	21–229	16	3.80 ± 0.31 (*e*)	94	18.3	156	12.2
1.80×10^6	6	330	45–330	20	3.91 ± 0.21 (*f*)	72	12.3	57	22.8
1.80×10^6	16	332	63–279	15	4.02 ± 0.29 (*g*)	77	11.1	109	21.2
Mean	.....	.....	.....	.....	3.87 ± 0.23	83	13.8	94	.....

where N=number of tumor cells t hours after inoculation, N_o=number of tumor cells inoculated, b=the regression coefficient of $\sqrt[3]{N}$ on t, t_o=the time (after inoculation) at which the extrapolated regression line assumes the value of $\sqrt[3]{N_o}$, *i.e.* where $\sqrt[3]{N}=\sqrt[3]{N_o}$.

The growth curves obtained after fitting equation (1) to the experimental points are represented in their semilogarithmic form (log N plotted against t) by the solid lines of text-figure 1. Considering the standard error of the cell-number determinations, the agreement between the experimental points and the computed curves is good.[6] The dotted parts of the curves are freehand drawings, corresponding to the earliest and latest growth periods, where equation (1) fails to represent the data.

For convenience of reference, the growth curves were divided into the following three parts:

1) The phase preceding the rise of the curves above the level of the inoculum will be referred to as the *latency period*. It is defined as the time that elapses before N becomes larger than N_o. An approximate measure of the length of the latency period is given by the value of t_o, obtained by the extrapolation of the regression line represented by equation (1). It is emphasized that t_o is only an approximate measure because it is not known whether or not the increase in cell numbers proceeds according to equation (1) between the end of the latency period and the first experimental point thereafter.

The extrapolated values of t_o are listed in table 3, column 10. It appears that the approximate length of the latency period decreases considerably with increasing inocula. With the largest inoculum used (40 million T-cells) there was no detectable latency period at all (the negative value of t_o in this case is an artefact due to the extrapolation).

A significant decrease in tumor-cell numbers during the latency period occurred only with the lowest dose of inoculum used (0.7 million T-cells). The experimental errors are great when working with cell numbers of this order of magnitude (see table 2) and it is not possible to decide whether the initial decrease is a real one, due to death or disappearance of the inoculated tumor cells from the peritoneal fluid, or is merely due to losses during the collection of the fluid that are proportionally greatest with the lowest dose of inoculum.

2) After the latency period has ended, the increase in the number of tumor cells can be well described by equation (1). The cube roots of the cell numbers fall approximately on a straight line during this period, which will be referred to as the *cube-root phase* (in analogy with the terminology used by Emerson (*25*) for a somewhat similar phase during Neurospora growth).

3) The cube-root phase proceeds until a T-cell number of about 10^9 is reached. When animal survival permitted observations to continue beyond this point, it was found that the cube roots do not fall on the same

[6] Almost equally good fit was obtained when using the general form of a parabola:

$$N-N_o=k(t-t_o)^f \qquad (2)$$

where N, k, and f are constants.

straight line any longer, and the curve approaches constant value (text-fig. 2). This last part of the growth curves (where tumor-cell numbers exceed 10^9) will be referred to as the *terminal phase.*

The rate at which the cube roots of tumor-cell numbers increased during the cube-root phase was different for the different growth curves of Series I (text-fig. 2). A numerical expression for this rate is given by the regression coefficients, listed in column 6 of table 3. The significance of the difference between the regression coefficients of the different curves was estimated according to the formula given by Simpson and Roe (*26*). The results are shown in table 4. It was found that the difference between the regression coefficients is significant for the three highest doses of inoculum (*b*, *c*, and *d*, column 6, table 3), while the difference between the two smallest inocula (*a* and *b*) is not significant. The increase rate of the cube roots is highest after the inoculation of 17.5 million cells, and it becomes lower when the inoculum dose is either increased to 40 million cells or decreased to 1.8 million.

TABLE 4.—*Comparison of the regression coefficients listed in table 3, column 6*

Regression coefficients compared (as labeled in col. 6, table 3)	t	P*	Regression coefficients compared (as labeled in col. 6, table 3)	t	P*
(*a*) and (*b*).....	0. 40	~0. 7	(*c*) and (*d*).....	7. 76	<0. 001
(*a*) and (*d*).....	4. 80	<0. 001	(*e*) and (*g*).....	0. 51	~0. 6

*P: probability that the difference is due to chance.

The fact that all the growth curves can be described by equation (1) (with the exception of the latency period and the terminal phase) has some practical consequences that can be summarized as follows:

1) *Curve fitting.*—The procedure of curve fitting is simple and convenient. Straight regression lines can be fitted by the method of least squares to the cube roots of tumor-cell numbers (text-fig. 2). The lines obtained can be easily transformed to represent the cell numbers in arithmetic or logarithmic units (text-fig. 1), and freehand drawing of the growth curves can be avoided.

2) *Mean generation time.*—The mean generation time for a population of unicellular organisms where the number of cells increases in geometric progression at a constant rate (exponential or logarithmic growth) is (*27*):

$$g=\frac{T_2-T_1}{\log N_2-\log N_1}\cdot\log 2 \qquad (3)$$

where g=mean generation time, N_2=number of organisms at time T_2, N_1=number of organisms at time T_1.

During the cube-root phase of ascites tumor growth, the changes in the number of free tumor cells can be described by equation (1). As cell number is not an exponential function of time, the mean generation time is not constant during any part of the cube-root phase. For an infinitely small instant (dt) the mean generation time is given by the following expression:

$$g_t=\frac{dt}{d\,(\log N)}\cdot\log 2 \qquad (4)$$

where N is the number of tumor cells and g_t is the mean generation time at time t during the cube-root phase.

From equation (1),

$$\log N = 3 \log [b(t-t_o) + \sqrt[3]{N_o}] \tag{5}$$

Hence,

$$\frac{d(\log N)}{dt} = \frac{3}{\ln 10} \cdot \frac{b}{b(t-t_o) + \sqrt[3]{N_o}} \tag{6}$$

$$\frac{dt}{d(\log N)} = \frac{\ln 10}{3}\left(t - t_o + \frac{\sqrt[3]{N_o}}{b}\right) \tag{7}$$

From equations (4) and (7):

$$g_t = 0.231\left(t - t_o + \frac{\sqrt[3]{N_o}}{b}\right) \tag{8}$$

or, expressing g_t as a function of tumor-cell number at time t:

$$g_t = 0.231 \frac{\sqrt[3]{N}}{b} \tag{9}$$

Expressions (8) and (9) are the equations of a straight line. Mean generation time increases thus linearly with the time that elapsed since growth has started ($t-t_o$) and also with the cube root of the tumor-cell number ($\sqrt[3]{N}$). In text-figure 3, the theoretical values of g_t are plotted against time for the growth curves of Series I. As follows from equation (8), the slope of the straight lines obtained is the same in all four cases while their intercept with the time-axis varies.

It should be pointed out that the formulas for mean generation time apply only if it can be assumed that all cells in the population are viable. The expressions would remain essentially the same, although with a different numerical constant, if a constant proportion of cells died in each generation (*27*). If an increasing proportion of cells should die in subsequent generations, the formulas would have to be modified. As will be shown below, in connection with the findings on cell viability, there is no evidence for an increasing death rate and the percentage of dead cells was very low during the entire growth period of the Ehrlich ascites tumor. Assuming that formulas (8) and (9) are thus reasonable approximations of the mean generation time for any given instant during the cube-root phase, it must still be emphasized that they indicate the time needed for the *average* cell in the population to divide into two. No certain inference can be made as to the actual generation times of the individual cells, as nothing is known of the individual differences from cell to cell. Increase of mean generation time might mean that the time needed for division increases uniformly in the whole population, but it is also possible that a number of cells fail to divide at all and that their proportion increases with time. The latter possibility is not too probable, however, as an increasing failure to undergo division could be expected to result in an increasing proportion of degenerating or dying cells, which was not the case. While these uncertainties make direct inferences with regard to the generation time of individual cells difficult, g_t is nevertheless a valuable parameter of ascites tumor growth.

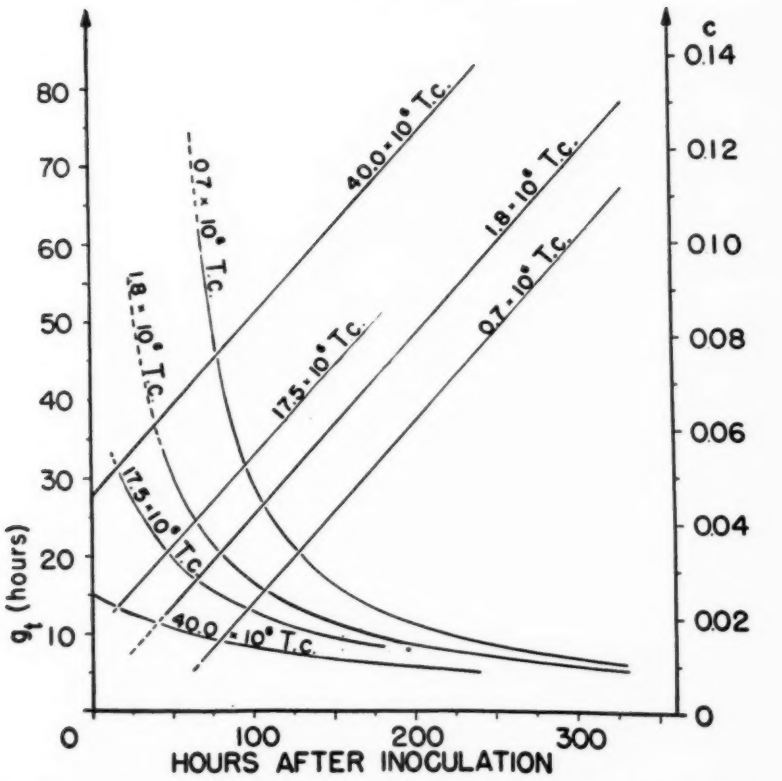
3) *Changes in growth rate.*—The relative rate of tumor-cell multiplication at any instant can be measured by the velocity coefficient, c, which denotes the number of new cells produced by the average cell in a unit of time.

$$c = \frac{dN}{dt} \cdot \frac{1}{N} \tag{10}$$

Multiplied by 100, c represents the percentage multiplication rate. From equations (1) and (10) it can easily be shown that c changes in the following way during the cube-root phase:

$$c = \frac{3b}{\sqrt[3]{N}} = \frac{3b}{b(t-t_o) + \sqrt[3]{N_o}} \tag{11}$$

A comparison of equations (9) and (11) shows that c is a reciprocal function of mean generation time, g_t, as could be expected (*27*).



TEXT-FIGURE 3.—Changes of the mean generation time and the relative multiplication rate of Ehrlich ascites tumor cells during the cube-root phase of their growth. The straight lines represent mean generation time at any given instant (g_t), while the curves denote relative multiplication rate $\left(c=\frac{dN}{dt}\cdot\frac{1}{N}\right)$. The four curves correspond to the four growth curves of text-figures 1 and 2. The dose of inoculum is indicated. The dotted parts of the curves are extrapolations to t_o.

Text-figure 3 shows the changes of c during the cube-root phase in the experiments of Series I. It will be seen that the rate of cell multiplication is maximal in the earliest stages of the cube-root phase and decreases thereafter, at first very abruptly and later increasingly slowly. The initial maximum growth rate is given by the following expression:

$$c_{max}=c_{t_o}=\frac{3b}{\sqrt[3]{N_o}} \qquad (12)$$

The reciprocal of expression (12), multiplied by ln 2, gives the minimum generation time (g_{t_o}) at the beginning of the cube-root phase.[1]

[1] The earliest parts of the g_t and c curves, between t_o and the first experimental points thereafter, are extrapolations (*cf.* the dotted parts of the curves in text-figure 3). It is probable that the cube-root phase does not start exactly at t_o, but at a somewhat different point of time. If this were so, maximum growth rate and minimum generation time would not depend directly on N_o (the dose of inoculum), but on the actual value of N at the beginning of the cube-root phase. This value is not known, however, and t_o and N_o can be assumed to be reasonable approximations.

If b were the same for all growth curves, maximum growth rate and minimum generation time would depend only on the cube root of the inoculum dose, and growth would start at a higher rate with smaller than with larger inocula. For this case it would also follow [from equations (9) and (11)] that different Ehrlich ascites tumors arising from different inocula grow at the same rate when they are of the same size (*i.e.*, contain the same number of tumor cells). However, it has been shown above (*cf.* column 6, table 3) that b is actually different for the different growth curves, and is highest for an inoculum of medium size (17.5 million cells). The differences in the values of b are not great enough to modify the fact that larger inocula start growing at a slower rate than smaller ones (text-fig. 3), but they are sufficiently great to result in important differences in the relative growth rate and the instantaneous mean generation time of different ascites tumors of the same size induced by greatly different inocula.

B. Cell Viability

Using the procedure of Schrek (*15*), the percentage of eosin-stained cells ranged between 1 and 7 percent (with an average of 3.9 percent) during the entire course of growth in Series I. There was no correlation between the percentage of stained cells and the time after inoculation. It is concluded that the great majority of cells from Ehrlich ascites tumors are viable during the entire growth period under the conditions used. It was not considered necessary to correct for the presence of dead cells when computing the total number of tumor cells for the various points of the growth curves, as the percentage of nonviable cells is negligible compared to the other errors involved.

With regard to Schrek's method of counting unstained cells, it has been stated recently (*28*) that unstained cells are viable, while not all stained cells are necessarily dead. If this is correct, the percentages of dead cells given for the Ehrlich ascites tumor may therefore be considered as maximum values. The same communication (*28*) also reports studies on the ascites form of Sarcoma 37, where the vast majority of the cells has been found to remain unstained when using fresh ascitic fluids.

C. Cell Volume

Average cell volume was determined by the hematocrit method at regular intervals, simultaneously with the experiments of Series I. As the various non-tumor cells occurring in the peritoneal exudate are generally smaller than the Ehrlich tumor cells (*5*), an increasing admixture of such cells will tend to lower the average cell volume. For this reason, only ascitic fluid containing at least 80 percent of tumor cells was used for the present studies. The average cell volume obtained is still slightly lower than the average volume of tumor cells alone would be. There is some preliminary evidence to indicate that the average volume of non-tumor cells occurring in the peritoneal fluid is minimally about one-fourth that of the tumor cells. A 20 percent admixture of such cells would lower the average cell volume by 15 percent. As no fluids containing more than 20 percent non-tumor exudate cells were used, the average cell volume obtained in the present experiments is maximally 15 percent lower than the average tumor-cell volume. Also, there is

often a slight admixture of erythrocytes, which tends to counteract the lowering effect of cells from the exudate, since the volume of erythrocytes is added to the packed column of the hematocrit while the number of red cells is ignored in the calculations. This effect is probably a very small one, however, as ascitic fluids containing considerable numbers of erythrocytes were not used for these determinations.

Twenty-one determinations of average cell volume were carried out, using ascitic fluid from 52 animals. The mean of all the determinations was 1903 μ^3 with a variation coefficient of 15 percent. For spherical cells this corresponds to an average cell diameter of 15.4 μ.

There was no correlation between average cell volume and time after inoculation. It should be emphasized, however, that the necessity of discarding all ascitic fluid with a tumor-cell concentration of less than 80 percent meant discarding the material obtained at the earliest and most rapid stages of growth. The proportion of tumor cells increased to 80 percent only when their total number was already 90 to 100 million cells or higher (text-figs. 7 and 8b). It is thus concluded that there was no change in average cell volume after this stage had been reached, and up to the attainment of the final maximum population.

D. Mitotic Cycle

If it could be assumed that the majority of cells undergo mitosis, the mean generation time (g_t) would indicate the length of one interphase+mitosis of the average tumor cell. As pointed out above, this assumption was not proved. It was shown, however, that the percentage of dead cells does not increase with time. If an increasing proportion of tumor cells cease to undergo mitosis during growth, it could be reasonably expected that this change would be somewhat reflected by an increase in cell mortality. As this was not the case, some calculations were made on the assumption that the great majority of the tumor cells undergo mitosis during the entire cube-root phase (for which phase g_t has been calculated). This also assumes that g_t represents the total length of one interphase+mitosis of the average cell.

Since g_t is given by equation (8) or (9) for the cube-root phase, a knowledge of the mitotic index would make it possible to calculate the average duration of mitosis. The mitotic index was therefore determined for the cube-root phase of two growth curves for Series I. The results are given in table 5. Mitotic times (length of mitosis) have been calculated according to Crick's formula, as given by Hughes [(*29*) p. 89].

The mitotic indexes tend to decrease with time (table 5). The calculated mitotic time varies between 34 and 132 minutes, with a possible tendency to increase in the later stages. The mean of the whole material gives 64 ± 7 minutes as the average length of one mitosis. This value is consistent with the findings of others for various solid and ascites tumors (*3*, *30–32*) and is slightly longer than the values reported for various normal proliferating mouse or rat tissues (*29*, *33–35*).

The probable prolongation of mitotic time during the later stages of

TABLE 5.—*Mitotic index and calculated average length of mitosis during the cube-root phase of two growth curves (Series I)*

Dose of inoculum: 17.5×10^6 *T*-cells				Dose of inoculum: 1.8×10^6 *T*-cells			
Hours after inoculation	Average mitotic index ± S. E.	Mean generation time (*hours*)	Average length of mitosis (*minutes*)	Hours after inoculation	Average mitotic index ± S. E.	Mean generation time (*hours*)	Average length of mitosis (*minutes*)
13	3. 26±0. 50	12. 6	34. 2	45	4. 91±0. 53	12. 4	48. 0
20	3. 11±0. 21	14. 0	36. 0	67	3. 17±0. 34	17. 0	45. 0
41	2. 95±0. 20	18. 6	46. 0	89	2. 67±0. 24	22. 8	50. 8
65	3. 39±0. 26	24. 4	68. 0	112	2. 48±0. 19	27. 4	56. 0
86	1. 72±0. 26	29. 0	42. 0	159	2. 00±0. 18	38. 9	63. 0
110	2. 56±0. 20	34. 8	74. 4	209	2. 46±0. 27	50. 5	103. 0
140	2. 64±0. 20	41. 7	92. 0	258	2. 54±0. 28	62. 0	132. 0
184	1. 10±0. 12	52. 2	49. 0	330	1. 29±0. 20	78. 2	80. 0
Mean			55. 2	Mean			72. 2

growth somewhat parallels the recent results of Browning *et al.* (*36*) who studied *in vitro* cultures of *Tetrahymena geleii*. Growth of this protozoan was characterized by continuously increasing generation time. Division time was constant and independent of generation time up to about ⅔ of the maximum population. Beyond this point there was an increase of division time. No definite prolongation of mitotic time could be demonstrated in our material until a population of about 600×10^6 cells was reached—a value nearly half that of the approximate maximum population.

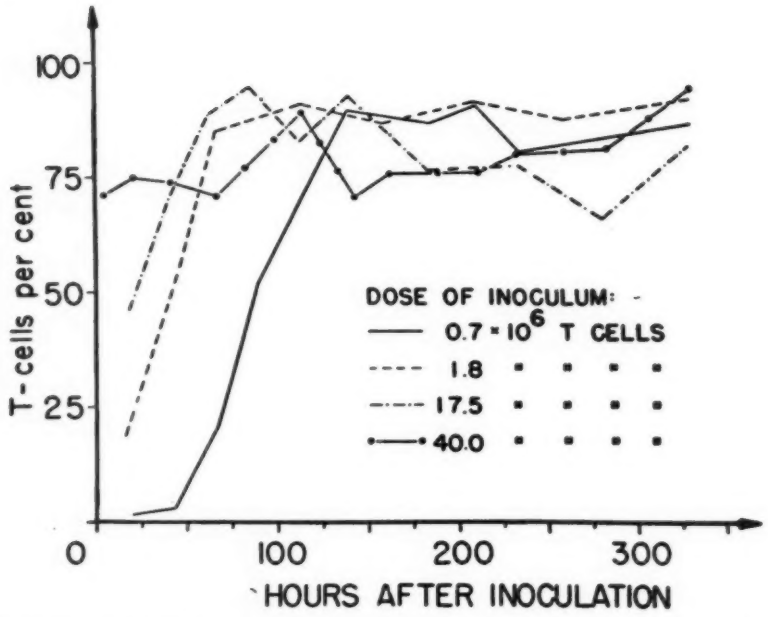
E. Changes in Number of Non-Tumor Cells and in Amount of Peritoneal Fluid During Ascites Tumor Growth

In previous papers (*5*, *6*) data have been presented on the cellular composition of the peritoneal fluid at various intervals after the inoculation of different numbers of Ehrlich ascites tumor cells. Generally, in the early stages of growth the percentage of non-tumor cells (*n*-cells) has been inversely related to the number of viable tumor cells injected. The proportion of *n*-cells was much higher after small inocula than after large ones, and the time needed for the tumor cells to reach the condition of a "nearly pure culture" was much longer for small than for large inocula. This seemed to indicate that an inflammatory reaction occurs after inoculation, the intensity and duration of which is inversely related to the dose of inoculum. The possibility remained, however, that this apparent inflammatory reaction is in reality only due to a dilution of the injected tumor cells with non-tumor cells, pre-existent in the peritoneal fluid of the host. This would produce an effect consistent with the apparent inverse relationship between tumor-cell dose and percentage of non-tumor cells.

The present data could be used to calculate the changes in the total number of *n*-cells in the course of ascites tumor growth, since the total number of cells and the percentages of tumor cells and non-tumor cells were determined for each point of each growth curve. The results are given in text-figures 4 and 5. The previously established inverse relationship between dose of inoculum and percentage of tumor cells in the early stages is again well demonstrated.

In text-figure 5 the logarithms of the numbers of non-tumor cells (*n*-cells) are plotted against time after inoculation. It appears that shortly after inoculation the total number of *n*-cells is higher for large than for small *T*-cell inocula. Furthermore, *n*-cells increase in number during the course of ascites tumor growth. This increase starts sooner after relatively large inocula than after smaller ones.

These results suggested that there may be a certain number of *n*-cells present in the peritoneal fluid before inoculation, and that this number is not negligible compared with the smaller doses of inocula. The *T*-cell inoculum would be diluted with these cells, and the percentage of *T*-cells lowered. This lowering must then be inversely related to the number of *T*-cells inoculated. Subsequently, the tumor cells start to multiply and soon attain the "nearly pure culture" stage, with 70 to 90 percent of tumor cells. After this point, there is a noticeable absolute increase in the number



TEXT-FIGURE 4.—Changes in the percentage of free tumor cells in the peritoneal fluid after the inoculation of four different cell doses. The data were obtained from the same experiments as shown in text-figures 1–3.

of n-cells (*cf.* text-figs. 4 and 5) that seems to be proportional to the increase of T-cells, holding the percentage composition of the fluid approximately constant.

Comparison of text-figures 4 and 5 suggested a more rigorous study, to try to elucidate the laws governing change in the number of non-tumor cells in the peritoneal fluid during the course of ascites tumor growth. A simple working hypothesis was formulated and the agreement between experimental data and hypothesis was tested as follows.

Working hypothesis

It was assumed that normal untreated mice of a certain strain, weight, and sex have a fixed amount of peritoneal fluid with a volume V_o. The total number of free cells in the entire fluid is approximately constant, and will be denoted by the symbol e_o. During ascites tumor growth, the amount of fluid increases proportionally to the number of free tumor cells. This newly formed fluid is added continuously to V_o. If the number of ascites tumor cells at any given time is designated by a, it can be written according to the hypothesis:

$$V = p \cdot a + V_o \qquad (13)$$

where V is the total volume of peritoneal fluid at time t, a is the number of tumor cells at time t, p is a constant, and V_o is the initial amount of peritoneal fluid in the normal mouse.

It was further assumed that the peritoneal fluid formed during the growth of the tumor cells contains a constant concentration of non-tumor cells. If the fluid increases

in proportion to the number of tumor cells, as assumed above, the number of non-tumor cells will also increase in the same proportion. The non-tumor cells that appear in the peritoneal fluid during this process are being added to the number of *n*-cells already there. This hypothesis can be expressed in the following way:

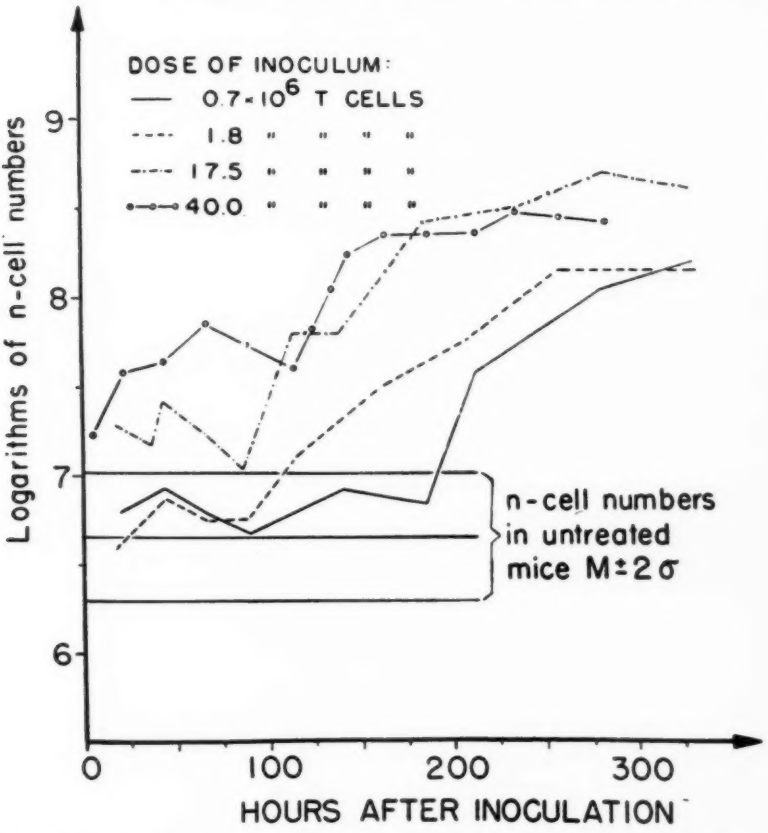
$$e = k \cdot a + e_o \qquad (14)$$

where e is the total number of non-tumor cells in the peritoneal fluid at time t, a is the total number of tumor cells in the peritoneal fluid at time t, k is a constant, and e_o is the number of non-tumor cells pre-existing in the peritoneal fluid of the untreated normal mouse.

Experimental

To test hypotheses (13) and (14), the following procedures were carried out:

a) *Determination of* e_o.—To estimate the number of free cells in the peritoneal fluid of normal mice, the abdominal cavity of 10 untreated animals (weighing about 20



TEXT-FIGURE 5.—Changes in the total number of non-tumor cells (*n*-cells) in the entire peritoneal fluid during the growth of the Ehrlich ascites tumor. Four different inocula of tumor cells were used. The data were obtained from the same experiments as shown in text-figures 1–3 (Series I). The number of *n*-cells present in the peritoneal fluid of the untreated normal mouse (with a body weight of about 20 grams) is indicated (mean $\pm 2\sigma$).

grams each) was rinsed with physiological saline in the same way as in the experiments with ascites tumors. The cells were counted in the pooled rinsing fluids for each mouse. Their total number in the entire peritoneal fluid was 4.8 million on an average, with a standard error of 0.6 million. This value corresponds to e_o in equation (14).

The cells could be classified as lymphocytes, mesothelial cells, histiocytes, and, to a small extent, polymorphonuclear leukocytes and mast cells. The proportions in which these different cell types occur was studied earlier (*5*). The average n-cell number $\pm 2\sigma$ (in logarithmic units) in normal mice has been indicated in text-figure 5.

b) *Determination of* V_o.—Five untreated normal mice were punctured with a capillary glass pipette and small amounts of peritoneal fluid were collected. The average concentration of cells in these fluids was 39.8×10^6 cells per ml. If the total number of cells (as obtained from the experiment described in the previous paragraph) is divided by the cell concentration, the amount of peritoneal fluid is obtained. Thus the volume of fluid in the peritoneal cavity of the untreated adult mouse (body weight about 20 grams) is about 0.12 ml. This value corresponds to V_o in equation (13).

c) *Determination of the amount of peritoneal fluid during ascites tumor growth.*—The total number of cells in the peritoneal fluid of the mouse has been determined, by the rinsing technique, for the entire course of ascites tumor growth. If the cell concentration (number of cells per milliliter) were also known, the amount of fluid could be calculated ($V = \frac{N}{conc.}$ where V is the volume of peritoneal fluid in ml., N is the total number of cells in the entire fluid, and *conc.* is the number of cells per ml.). To secure the necessary data, 40 mice were inoculated with 1.8 million tumor cells in two different experiments. The physiological age of the inoculum was 6 days. Peritoneal fluid was collected with a glass capillary at various intervals after inoculation and the concentration of cells was determined. Each mouse was used only once. The percentage of tumor cells was differentially counted for each sample on stained smear preparations, and the concentration of tumor cells per ml. computed. The total number of tumor cells at corresponding points of time was then read from the growth curve obtained in Series I for the same inoculum (text-fig. 1). Dividing total number by concentration, the amount of fluid was calculated. Obviously, the variability among animals will introduce an error, as the total number of cells and the concentration were determined on different individuals. However, good reproducibility of the growth curves justifies the procedure used.

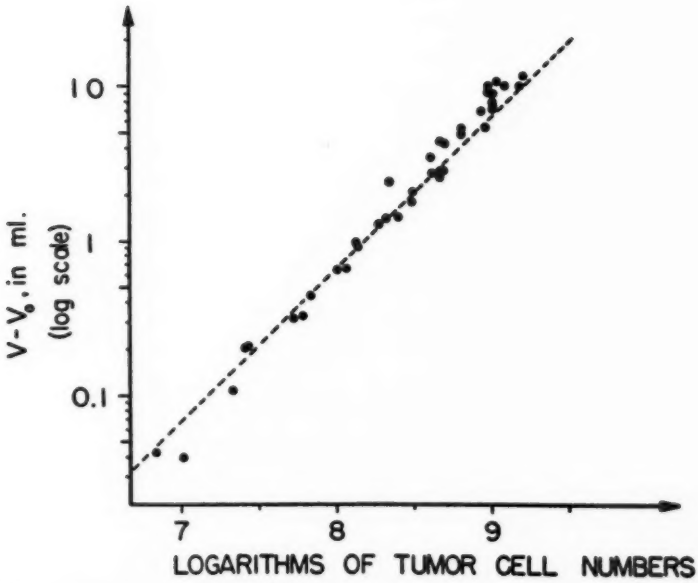
Comparison of experimental data and working hypotheses

a) *Amount of peritoneal fluid* (V).—Equation (13), indicating the hypothetical increase of peritoneal fluid during ascites tumor growth, was plotted as a curve in the following way:

The initial average amount of fluid, V_o, has been determined experimentally as described above, and was found to be 0.12 ml. per 20-gram mouse. The constant p denotes the proportion in which the increase of peritoneal fluid follows the multiplication of the tumor cells. For well developed ascites tumors with a fluid volume much larger than V_o, p approximates the ratio between fluid volume and number of tumor cells, as V_o is becoming negligible. Data were available from previous work (*13*) showing that the average concentration of tumor cells was 130.9 ± 4.3 million cells per milliliter in 65 different ascitic fluids, all collected close to the median survival time. Median survival time represents a far-advanced stage of ascites tumor growth, with several milliliters of fluid. V_o can therefore be neglected, and $\frac{1}{131 \times 10^6}$ can be taken as the approximate value of the constant p.

After the constants V_o and p have thus been determined empirically, equation (13) could be represented by a curve. As deviations from a straight line are easiest to detect, it was decided to plot log $(V - V_o)$ against log a (text-fig. 6).

It appears that equation (13) agrees fairly well with the data. Possible slight deviations are indicated by some early values that are too low and by the group of



TEXT-FIGURE 6.—Relationship between the amount of ascitic fluid and the number of free tumor cells. The ordinate represents (on a logarithmic scale) the amount of peritoneal fluid after subtraction of the initial volume of fluid before inoculation (V_o). Thus it denotes the amount of fluid formed after the tumor cells were inoculated. The abscissa indicates the logarithms of the numbers of tumor cells. The dotted line is the theoretical relationship expressed by equation (13) while the black points represent the experimental data.

higher values at the end of the line. Further experiments are necessary to determine what significance can be attributed to these deviations. For practical purposes equation (13) can be accepted as an approximate description of the relationship between fluid volume and number of tumor cells, at least for the Ehrlich ascites tumor.

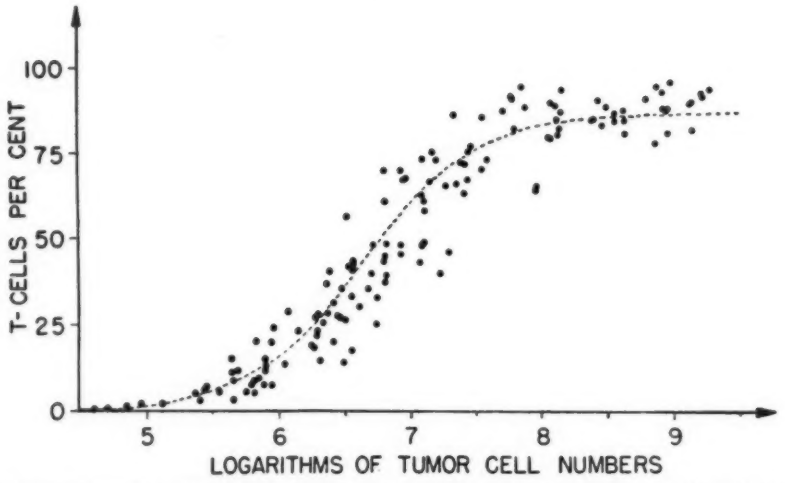
b) *Number of non-tumor cells* (e).—Data from all experiments of both Series I and II were collected and tabulated. The greatest number of values was available for the inoculum dose of 1.8×10^6 tumor cells and these will be given below in some detail. The data were arranged in the order of the time (after inoculation) when they were obtained. For each point of time, one or several values were available for the total number of tumor cells in the peritoneal fluid, a; total number of non-tumor cells in the peritoneal fluid, e; and the percentage of tumor cells, b. In text-figure 7, b was plotted against log a. The values obtained from the same mouse at the same time were plotted against each other.

The percentage of tumor cells, b, is obviously:

$$b = \frac{100a}{a+e} \tag{15}$$

According to the hypothesis represented by equation (14), b can be expressed as a function of a only, in the following way (from equations 14 and 15):

$$b = \frac{100}{1+k+\frac{e_o}{a}} \tag{16}$$



TEXT-FIGURE 7.—Relationship between the percentage of tumor cells, b, and the logarithms of their total number (log a). The data have been obtained at different times after the inoculation of 1.8 million Ehrlich ascites tumor cells. The dotted line represents the theoretical equation (16). The points are experimental data, each of which was obtained from a different mouse.

On the basis of equation (16) a theoretical curve of b against a can be plotted and a study made of how well the curve fits the experimental points.

The constant k could be determined in the following way: As the value of e_o is about 4.8 million, the quotient $\frac{e_o}{a}$ in equation (16) approaches zero when a increases to high values during ascites tumor growth. At the same time b approaches a maximum limit. It can be written:

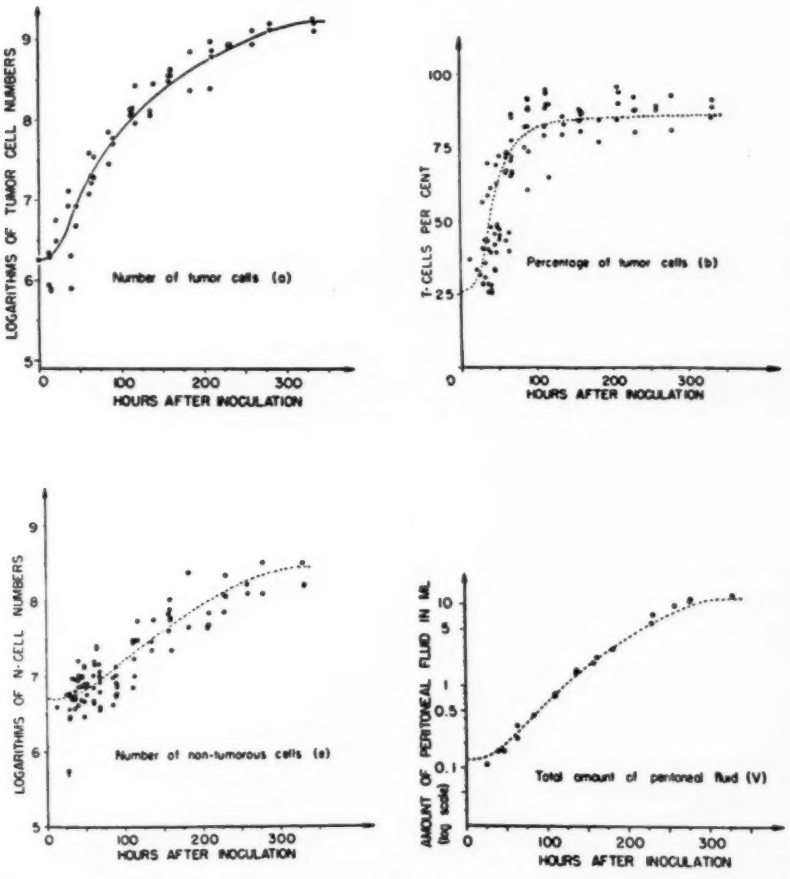
$$\lim_{a \to \infty} b = B = \frac{100}{1+k} \qquad (17)$$

It appears from text-figure 7 (see also text-fig. 4), in conformity with expression (17), that the empirical values of b tend to reach a constant maximum when the total cell number, a, becomes very high. When log a is higher than 8, b shows only random fluctuations. All experimental values of b with a log a abscissa higher than 8 were therefore added and the average calculated. This average was 86.2 percent of tumor cells and it can be considered to lie close to b's theoretical limit, B. It was therefore substituted instead of B in equation (17), and k was calculated. The numerical value of k is then 0.1597 and it denotes, according to the working hypothesis, the number of new n-cells that appear in the peritoneal fluid when the number of T-cells increases with one unit. The number of n-cells would thus increase by 16 when 100 new T-cells are formed.[8]

[8] It has been pointed out to the authors that the same results would be obtained if the apparent increase of n-cells during ascites tumor growth were due to a consistent under-reading of tumor cells in differential counts. The k of 0.16 could then be interpreted to mean that for every 100 tumor cells actually counted, an additional 16 that were counted as normal were in fact tumor cells. In that case, the real percentage of tumor cells would approach 100 as time progressed. In the well established ascites tumors (containing 400 to 1,000 million cells) that are most often used for experiments, it would actually be between 98.8 and 99.5 percent [from equation (15), if $c = c_o$]. It has been found previously, however, that about 10 percent of *small inflammatory cells* consistently contaminate the well-developed Ehrlich ascites tumor (*13*). These cells—polymorphonuclear leukocytes, lymphocytes, and small histiocytes—are most unlikely to be confused with tumor cells in this particular tumor with its large, predominantly tetraploid, and very characteristic cells. There is an additional admixture of 2 to 6 percent of mononuclear cells, some of which could conceivably be tumor cells, although not counted as such. This error would, however, only shift the value of k, but equation (14) and the conclusions derived from it could be maintained unaltered.

After k has been calculated, the theoretical curve of b against log a could be drawn (using equation (16) for the calculation of the theoretical b values). This theoretical curve is represented by the dotted line in text-figure 7. There seems to be remarkably good agreement between the hypothetical curve and the experimental values. The differences between the corresponding theoretical and empirical values of b were calculated, and the mean difference was found to have no statistical significance ($n=139$, $\overline{d}=-1.41, \epsilon_{\overline{d}}=0.72, t=1.96, 0.1>P>0.05$).

The agreement between hypothesis and experiment can also be demonstrated when plotting the data against time, as in text-figure 8. The *empirical* growth curve after inoculation of 1.8×10^6 cells (text-fig. 8,*a*) was plotted against time and the correspond-


LOGARITHMS OF TUMOR CELL NUMBERS
Number of tumor cells (a)
HOURS AFTER INOCULATION
T-CELLS PER CENT
Percentage of tumor cells (b)
HOURS AFTER INOCULATION
LOGARITHMS OF N-CELL NUMBERS
Number of non-tumorous cells (e)
HOURS AFTER INOCULATION
AMOUNT OF PERITONEAL FLUID IN ML. (log scale)
Total amount of peritoneal fluid (V)
HOURS AFTER INOCULATION

TEXT-FIGURE 8.—Comparison of experimental data and working hypotheses with regard to changes in the percentage of tumor cells, b; total number of non-tumor cells in the exudate, e; and volume of peritoneal fluid, V, with time after the inoculation of 1.8 million Ehrlich ascites tumor cells. The left upper curve shows the empirical growth curve of the tumor cells (a). The three other figures show the corresponding changes of b, e, and V. The hypothetical curves [according to equations (13) and (14)] are represented by the dotted lines, while the black points indicate the actual experimental data.

ing *theoretical* values of e, b, and V were calculated from equations (14), (16), and (13). The values of a were read from the empirical growth curve.

There is good agreement between hypothetical expectations and experimental data in all three cases, whether studying the percentage of tumor cells (b), the total number of n-cells (e), or the amount of peritoneal fluid (V).

It seems safe to conclude that the two simple hypotheses expressed by equations (13) and (14) describe the experimental data satisfactorily and without necessitating further assumptions. Small deviations may exist, especially in the earliest and latest stages of growth, but for most of the growth period it will not be far from the truth to state that the accumulation of peritoneal fluid and the increase of the number of non-tumor cells in the exudate are directly proportional to the multiplication of ascites tumor cells. It should be emphasized that this has been shown for the Ehrlich ascites tumor only, and has been principally based on results from an initial inoculum of 1.8 million cells. It does not necessarily hold for other cases.

F. Infiltration of Solid Intra-abdominal Host Tissues With Tumor Cells

The results of histological studies are shown in table 6. Column 3 shows the last point of time after inoculation at which no growth of tumor cells was detectable in the mesenterium, omentum, pancreas, parietal and visceral peritoneum, and fatty tissue. Column 4 shows the first time when groups of tumor cells were found within the tissues studied. There are no data available for the interval between the points of "no infiltration" (col. 3) and "detectable infiltration" (col. 4). These points represent, therefore, the limits between which tumorous infiltration of the host tissues begins.

The histological material was classified as belonging to the "no infiltration" group when only occasional T-cells could be seen attached to the peritoneal surface. There was usually no difficulty in classifying the material in one group or the other, as infiltration started simultaneously at several sites. Figures 5 to 11 illustrate some of the observed stages of tumorous infiltration in the mesenteric fat and the pancreas.

TABLE 6.—*Data on the time (after inoculation) when tumorous infiltration of solid intra-abdominal tissues first appears*

(Ehrlich ascites tumor)

Number of T-cells inoculated	Age of inoculum (*days*)	Last point when no infiltration detectable (*hours after inoculation*)	Infiltration first detectable (*hours after inoculation*)
1	*2*	*3*	*4*
40×10^6	6	66	114
17×10^6	6	113	137
1.8×10^6	6	90	133
1.8×10^6	3	85	117
1.8×10^6	6	89	113
1.8×10^6	13	111	158
Average		92.3 (=3.85 days)	128.7 (=5.36 days)

The data in table 6 imply that, under the conditions of these experiments, tumor infiltration of the intra-abdominal tissues of the host is most likely to begin on the fourth or fifth day after inoculation. This is in agreement with our previous findings (*5*). There is no certain difference between the six different experiments, although the inoculated dose has been varied from 1.8 to 40 $\times$ 10^6 *T*-cells and the inoculum age from 3 to 13 days.

Considerable multiplication of free ascites tumor cells occurs even before the fourth day (*cf.* text-figs. 1 and 3). It is concluded that multiplication of ascites tumor cells can occur in the absence of, and independently from, solid tumor implants. This conclusion has been previously reached by Goldie and Felix (*7*) and by one of us (*5*, *6*).

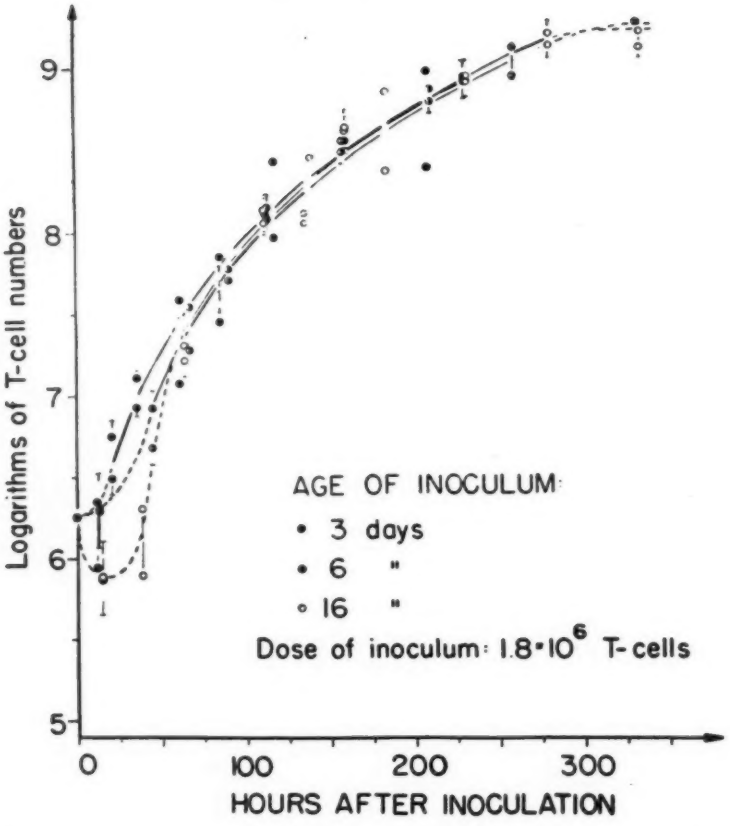
Results—Series II

The experiments of Series II were carried out in order to find whether variation in the physiological age of the inoculum was followed by any change in ascites tumor growth. Text-figures 9 and 10 show the growth curves obtained after the inoculation of cells 3, 6, and 16 days old. The dose of inoculated cells was kept constant (1.8 $\times$ 10^6). Text-figure 9 is plotted on a semilogarithmic scale (as in text-fig. 1), while text-figure 10 represents the cube roots of the cell numbers (corresponding to text-fig. 2). Regression data in table 3 were used for text-figure 10. The cube-root phase of the three growth curves is nearly identical. The regression lines are almost parallel (text-fig. 10) and the difference between the regression coefficients is not significant (table 4). There is some indication of possible differences in the length of the latency period; proliferation seems to start slightly later with the older inocula. It could not be decided on the basis of the present data whether this difference is due merely to chance. The question seemed worth further investigation in light of the well established fact that in cultures of unicellular organisms cell division starts later when older inocula are used (*37*, *38*). The experiment was therefore repeated. Three groups of mice, each containing 30 animals, were injected with cells 3, 6, and 16 days old, respectively, as in the experiment shown in text-figures 9 and 10. Pairs of mice were killed at 4-hour intervals for 3 days after inoculation. No consistent difference could be established between the three groups with regard to the length of the latency period, and it was concluded that variations in the physiological age of the inocula between 3 and 16 days have no discernible effect on the proliferation of Ehrlich ascites tumor cells.

Results—Series III

A. Growth Curves of the MC1M Ascites Sarcoma

The MC1M sarcoma is now carried in two independent lines in our laboratory. One is the original solid tumor, kept by subcutaneous transfers. This is called line SS and it has never been transplanted to the peritoneal cavity. The tumor is also kept in the ascites form, by intraperitoneal transfers of ascitic fluid (line A). Subcutaneous injections of



TEXT-FIGURE 9.—Increase of the total number of free Ehrlich ascites tumor cells in the entire peritoneal fluid of the mouse. The dose of inoculated cells was kept constant (1.8 million *T*-cells) while the physiological age of the inoculum varied (3, 6, or 16 days). Each point represents a different mouse. The standard error for single determinations ($\pm \sigma_s$) is indicated. The dotted lines are freehand drawings, while the black curves represent equation (1) fitted to the data.

ascites from line A produce solid tumors (called line AS) that are able to induce typical ascites tumors immediately after their first intraperitoneal inoculation—in contrast to tumors of the SS line, which can be transformed into ascites tumors only after they have been carried through a number of "fluid transfer generations" (*5*, *39*).

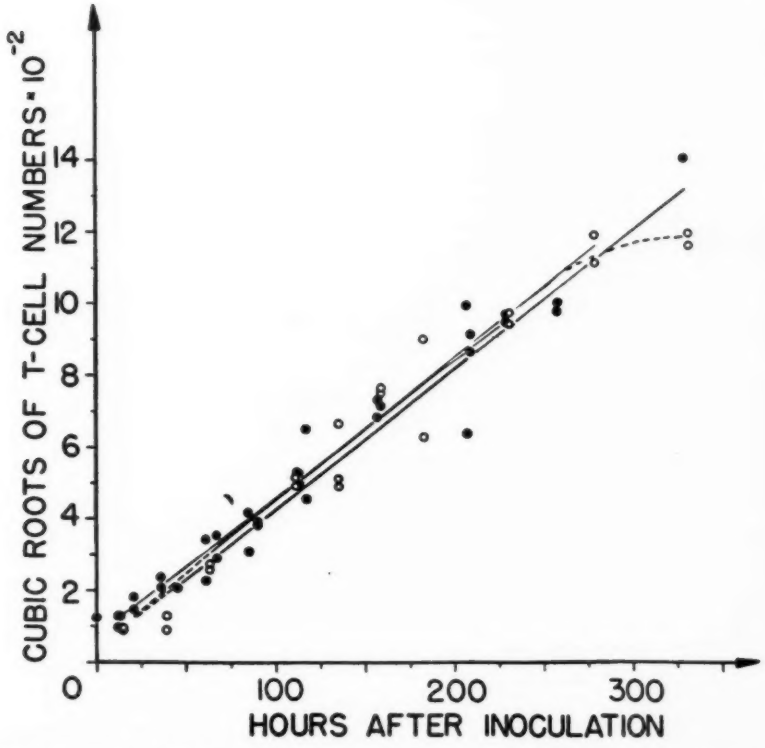
Free tumor cells of line A multiply rapidly after inoculation and attain a high concentration in the peritoneal fluid. At the same time the amount of ascitic fluid increases. The tumor cells are morphologically different from the Ehrlich ascites tumor cells in several respects [(*5*) figs. 3 and 4].

The same procedures were used as for the experiments in Series I. The size of the inoculum was varied while its physiological age was kept

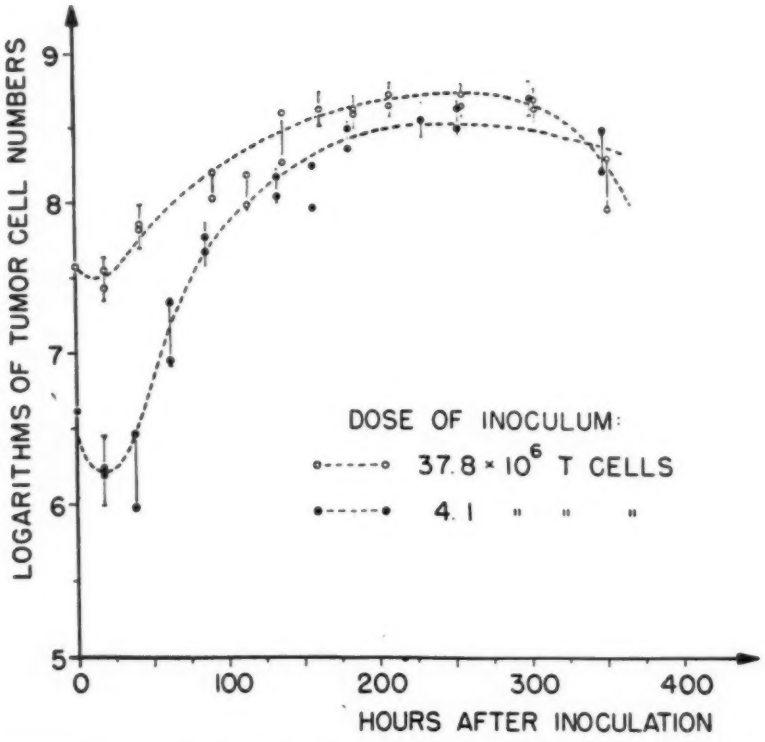
constant (14 days). Three inocula were used, containing 37.8, 4.1, and 0.45 million tumor cells.

Text-figure 11 shows the curves obtained with the two higher doses. With the lowest dose (0.45×10^6 *T*-cells) ascites was only obtained in a small proportion of the inoculated animals, while the rest developed solid tumors exclusively. This dose, therefore, appears to be below the "critical inoculum level" (*6*) necessary to induce ascites tumors in 100 percent of the animals.

The growth curves of text-figure 11 show some similarities to the curves for Ehrlich ascites tumors (text-fig. 1), but there are also important differences. As with the Ehrlich tumor, after an initial period in which the number of tumor cells remains constant or decreases, growth starts at a higher rate from the smaller than from the larger inocula. But cell multiplication slows and stops much earlier in the MC1M tumor. The high maximum populations of 1,000 to 1,500 million free tumor cells found to be characteristic for the Ehrlich ascites tumor are never reached with the MC1M sarcoma, for which the highest values are about 400 to



TEXT-FIGURE 10.—The same as text-figure 9, except that the ordinate now represents the cube roots of tumor-cell numbers ($\times 10^{-2}$) instead of the logarithms. The black lines are calculated regression lines, the dotted lines are freehand drawings.



TEXT-FIGURE 11.—Increase of the total number of free MC1M ascites sarcoma cells in the entire peritoneal fluid of C3H mice. The physiological age of the inoculum was kept constant (14 days). Each point represents a different mouse. The standard error for single determinations ($\pm\sigma_s$) is indicated. The curves are freehand drawings.

600 million. The mice are able to survive for 7 or 8 days after the number of free MC1M cells in their peritoneal fluid has attained the maximum, while a similar prolonged survival does not seem possible for animals with the maximum population of Ehrlich cells. During this last week of survival the MC1M ascites was observed to become more and more hemorrhagic, and subcutaneous and intra-abdominal solid tumors increased in

TABLE 7.—*Percentage of dead tumor cells during the growth of the MC1M ascites sarcoma*

(*Inoculum dose: 4.1 × 10⁶ tumor cells*)

Hours after inoculation	Percentage of eosin-stained cells (means of 2–7 determinations)	Hours after inoculation	Percentage of eosin-stained cells (means of 2–7 determinations)
84	0.4	254	1.2
132	0.3	302	36.4
230	0.6	326	27.7

size. Finally, shortly before death, there was a definite downward slope of the growth curves, indicating a decrease in the number of free ascites tumor cells. Such a decrease was never observed with the Ehrlich ascites tumor.

Determinations of cell viability by the eosin method showed that the percentage of dead cells increased markedly in these last stages. Table 7 gives the results pertaining to the growth curve obtained after the inoculation of 4.1 million MC1M cells.

When the cube roots of cell numbers were plotted against time it was found that they fall close to a straight line during the relatively short period of growth.

The changes in the percentage of tumor cells and the number of non-tumor cells were in all details similar to the findings reported above for the Ehrlich ascites tumor. The maximum percentage of tumor cells was 85 to 90 percent. The number of non-tumor cells could be well described by equation (14) at any given time. Concerning the amount of fluid, preliminary experiments indicate that the simple relationship expressed by equation (13) does not hold for the MC1M ascites tumor, as the cell concentration decreases during the terminal stationary phase, indicating an increase of the fluid volume in the absence of any increase of tumor cells. Simultaneously the erythrocyte content of the fluid increases markedly. The causes for the differences in the behavior of the MC1M and Ehrlich tumors are being further investigated.

B. Growth of the 3 Forms of MC1M Sarcoma Compared

To compare the growth of MC1M sarcoma in the original solid form (line SS), the ascites form (line A), and in the form of solid tumors produced by the subcutaneous inoculation of ascites (line AS), cell suspensions from SS and AS solid tumors were injected subcutaneously into 2 groups of 20 C3H mice each. The solid tumors that developed were measured daily with a caliper as soon as they became palpable and until the 20th day after inoculation. Three diameters were measured and the geometric mean calculated by the method proposed by Schrek (*21, 22*). The mean diameter was found to increase approximately linearly with time for both the SS and AS tumors. When least-squares lines were fitted to the data it appeared that the mean diameter of the SS tumors increases with 1.04 mm. per day on an average, while the corresponding figure was 1.11 mm. for the AS tumors.

As calculated from the two growth curves shown in text-figure 11, the cube roots of the numbers of ascites tumor cells increase at a rate of 2.90 and 2.58 $\sqrt[3]{N}$ per hour in the ascites (A) line of the MC1M sarcoma during the cube-root phase. The difference between the two curves was not significant ($P \sim 0.3$). If the ascites tumor is assumed to correspond to a solid tumor having the same volume as the total volume of all ascites cells, it can be shown [see equations (18) and (19), below] that the increase rates of $\sqrt[3]{N}$ correspond to a daily increase with 1.12 and 1.00 mm., respectively, of the diameter of a solid tumor. [The average cell volume

of the MC1M ascites sarcoma is very close to that of the Ehrlich ascites tumor, and therefore the same constant was used as in equation (19)]. This increase is about the same as the increase of the mean diameters actually obtained for the AS and SS lines of MC1M solid tumors. It is concluded that the present comparison has failed to reveal any major difference between the growth rates of the three lines. The ascites form of the MC1M sarcoma does not seem to possess a growth capacity that would exceed the growth rates of the corresponding solid tumors to any major degree, whether these were produced by subcutaneous inoculation of ascitic fluid, or by serial subcutaneous transfer of the original solid line.

Discussion

It will be attempted in this discussion to compare the data obtained on the multiplication of ascites tumor cells with the growth characteristics of solid transplantable mammalian tumors and of rodent embryos. Some features of the growth curves will be considered in the light of available information on the behavior of microorganisms in analogous cases.

A. Comparisons Between the Growth of Ascites Tumors, Solid Tumors, and Rodent Embryos

It has been found by Mayneord (*20*), Schrek (*21*, *23*) and others that the linear dimensions of certain subcutaneously transplanted solid tumors increase linearly with time. This is surprising in view of the current assumption that the multiplication of tumor cells is governed by the exponential law, *i.e.* that their number increases in geometric progression at a constant rate. Mayneord has shown, however, that the exponential law can be reconciled with these findings if it is assumed that only the cells near the surface of the tumor are in active division.

The location of the individual cells with respect to nutrient vessels is of major importance in the nutrition of solid tumors. This is reflected, for example, in their highly varying content of nucleic acids and proteins (*40*). Cells near the surface of the tumor and close to vessels have better access to nutrients and should grow at a higher rate than cells having to rely on diffusion over longer distances. In ascites tumors there are no such environmental differences. The cell-containing fluid is continuously mixed, no gradients of diffusion can exist, and all cells have the same amount of nutrients available at a given moment. In agreement with this, it has been found (*13*) that the great variation in nucleic acid content encountered in a solid tumor cannot be demonstrated if the same tumor grows in the ascites form.

If tumor-cell multiplication were governed by the exponential law, one could expect the growth curves of ascites tumors to be exponential if it can be presumed that all cells are undergoing division. The latter assumption has not been critically proved and it is possible that an increasing percentage of cells cease to divide, or divide at a slower rate, as time proceeds—thereby causing deviations from the exponential curve.

It would be a remarkable coincidence, however, if the growth curve obtained were of exactly the same type as the growth curves of solid tumors, provided that Mayneord's hypothesis is correct and the linear increase of solid tumor diameters is an effect of central necrosis. Since the great majority of ascites tumor cells are viable during the entire growth period and there is nothing that would correspond to central necrosis, one could reasonably expect ascites growth curves to be basically different from solid ones. This expectation was not verified by the present investigations, but the converse could be ascertained. A measure comparable to the diameter of solid tumors, namely the cube root of the number of ascites tumor cells, was found to increase linearly with time during the period of active growth. It was possible, furthermore, to compare the growth of the MC1M ascites sarcoma with the original solid form of this tumor and also with solid tumors produced by subcutaneous inoculation of ascitic fluid. No significant difference was found between the growth of the sarcoma in the solid or ascites form, as the cube roots of ascites cell numbers increased at a rate corresponding to the increase of the solid tumor diameter within the limits of error. It would be surprising if this qualitative and quantitative similarity of the growth curves of solid and ascites tumors were a mere coincidence. It is more probable that the course of tumor-cell multiplication *in vivo* is similar under both conditions and growth is not exponential but rather can be characterized by continuously increasing generation times. Furthermore, the linear increase of solid tumor diameters is not necessarily an effect of central necrosis, but may be the result of a cubic increase in the number of tumor cells.

The similarity of the growth curves for ascites and solid tumors facilitates further quantitative comparisons. Since the average volume of cells was found to remain constant during the major part of ascites tumor growth, it will be assumed that the total volume of all ascites cells (both tumorous and nontumorous) is comparable to the volume of a spherical solid tumor. On the basis of this assumption,

$$D=2\cdot\sqrt[3]{\frac{3}{4\pi}\cdot(a+e-e_o)v} \tag{18}$$

where D is the diameter of a solid tumor with the same volume as the total volume of all cells in the ascites; a, e, and e_o are the same symbols as in equation (14): a is the total number of ascites tumor cells, e the number of non-tumor cells in the exudate at a given time, and e_o the number of non-tumor cells pre-existent in the peritoneal fluid of the untreated normal mouse; v is the average volume of the ascites cells.

Using equation (14) for e and the empirical value of 1903 μ^3 for the average volume of cells in the Ehrlich ascites tumor, it follows:

$$D=0.01614\ \sqrt[3]{a}\ \text{(in millimeters)}. \tag{19}$$

Using equation (19), the regression coefficients of table 3 (column 6), showing the rate of increase of the cube roots of ascites tumor-cell numbers

per hour, can be transformed to represent the average linear increase per day (in millimeters) of the imaginary solid tumor assumed to correspond to the Ehrlich ascites tumor. The average of all regression coefficients listed in table 3, column 6, will then correspond to a daily increase with 1.50 mm. (range 1.15–1.85).

Obviously, these calculations represent only a rough approximation, as the increase rate of ascites cell numbers depends on the formation of new tumor cells and the appearance of new exudate cells in the peritoneal fluid, while the growth rate of a solid tumor is the result of tumor-cell multiplication, increase of stromal elements, and the counteracting influence of necrosis. The progressive increase in the number of non-tumor cells in the ascites may partly correspond to the stromal increase in the solid tumor, especially if the latter is counteracted by necrosis. In view of these difficulties and uncertainties in the comparison, it is surprising to find that the average increase of 1.5 mm. per day calculated from the growth of the Ehrlich ascites tumor is close to what Schrek has described as the mean linear growth constant for two anaplastic and rapidly growing solid rat sarcomas (*22*). The R39 sarcoma was found by Schrek to grow at an average rate of 1.78 mm. per day, while the Walker tumor 256 had a mean growth constant of 1.22 mm. per day. This agreement could be a pure coincidence, but it may also be the result of a fundamental similarity in the growth characteristics of anaplastic tumor cells, whether growing in the solid or ascites form.

In another paper (*24*) Schrek has compared the growth of three rat tumors with the growth of the rat embryo. This comparison was facilitated by the known fact (*41*) that the cube root of the weight of vertebrate embryos increases linearly with time. Calculating the cube root of the weight of the three rat tumors and plotting it against time, straight lines were obtained with a slope directly comparable to the slope of the corresponding line representing embryonic growth. Having made this comparison, Schrek found that the rate of embryonic growth is approximately equal to the average growth rate of R39 sarcoma, the most rapidly growing of the three neoplasms. In agreement with previous authors, he concluded that tumors do not exceed embryonic tissues in growth capacity.

It was interesting to compare the growth rate of the Ehrlich and MC1M ascites tumors to that of the mouse embryo. It can be calculated from the data of MacDowell *et al.* (*42*) that the mouse embryo grows at a rate of about 0.11 $\sqrt[3]{\text{gr}}$ per day. The cube root of the total weight of the cells in an ascites tumor is equal to the product of the cube root of their total volume and the cube root of the specific gravity. As the specific gravity of the cells is about 1.03, the cube root of this figure is close to 1 and the cube root of weight is approximated by the cube root of volume. If the diameter of the imaginary solid tumor representing the totality of Ehrlich ascites cells increases with 1.5 mm. daily, the cube root of the total cellular weight will then increase by about 0.12 $\sqrt[3]{\text{gr}}$ per day ($\sqrt[3]{\text{weight}} \approx \sqrt[3]{\text{volume}} = 0.806 \times \text{diameter}$).

This is almost the same figure as the one obtained for the mouse embryo. The MC1M ascites sarcoma was growing at a slower rate, about 0.085 $\sqrt[3]{gr}$ per day. As the Ehrlich ascites tumor is one of the most rapidly growing and most anaplastic mouse neoplasms known and has a transplantation history of more than half a century, it can be concluded, in agreement with previous authors (*43*, *24*, and others), that neoplasms can only approach but not exceed embryonic growth rate, even when they are proliferating at maximum speed.

Inoculum size and growth rate

The effect of inoculum size on the rate of linear growth of solid tumors was investigated carefully by Schrek (*23*). A slight but consistent decrease of the linear growth constant followed tenfold and hundredfold decreases of inoculum dose with three out of four rat tumors. As already mentioned, Schrek's linear growth constant is comparable to the slope of the line representing the increase of the cube root of the number of ascites tumor cells with time. The numerical expression of this slope is given by the calculated regression coefficients. The four growth curves of Series I, where the size of inoculum varied, permit a comparison with Schrek's findings on solid tumors. The regression coefficients for these four curves (listed in table 3, column 6) show that the cube roots increase at the highest rate after the inoculation of 17.5 million cells. The rate of increase is smaller when the inoculum dose is either increased to 40 million or decreased to 1.8 million cells. The differences are statistically significant. These findings do not disagree with Schrek's results for solid tumors, as the doses of inoculum he used were probably well below 17.5 million cells. Furthermore, they are comparable to the observations of Earle *et al.* (*44*) who found that the highest rates of proliferation in tissue cultures are obtained with an intermediate inoculum dose. If the optimum dose was greatly changed proliferation invariably decreased, whether inoculum size was enlarged or diminished.

Inoculum size and latency period

Various authors (*21*, *45*, *46*) have found that the "latency period" of solid tumors—the time that elapses before the tumors reach palpable size—is inversely related to the dose of inoculum. In comparing this finding to the present results on ascites tumors, it should be noted that "latency period" has a different meaning for the growth of ascites tumors than for solid tumors. In ascites tumors it means the time elapsing before the number of free tumor cells rises above the level of the inoculum. The latency period of solid tumors is partly dependent on the time before the inoculated cells start proliferating—and this part is comparable to the latency period of ascites tumors—and partly on the subsequent growth rate of the cells until the tumor reaches palpable size.

It has been demonstrated in the present investigation that the latency period of ascites tumors is inversely related to inoculum size. Furthermore, the rate of increase of $\sqrt[3]{a}$, the dimension comparable to the diameter of solid tumors [*cf.* equation (18)], diminishes when the inoculum dose is

decreased below an optimum level. (This optimum is rather high, well above the usual amount of solid tumor transplanted.) Both latency period and subsequent growth of the linear dimension are thus inversely related to inoculum dose in ascites tumor growth. This agrees with the well-known fact that the latency period of solid tumors increases with decreasing inoculum doses, and indicates that such an increase may be partly due to a prolongation of the period before the inoculated cells are able to proliferate, and partly to a subsequent reduction of growth rate.

B. Comparisons Between the Multiplication of Ascites Tumor Cells and of Microorganisms

These comparisons are limited to a few analogies, mainly because the present studies were performed on tumor cells growing *in vivo*, while most investigations on the growth of microorganisms are based on cultures *in vitro*.

The lag phase during the growth of microorganisms is somewhat comparable to the latency period of ascites tumors. It has been found that the lag phase of bacteria (*27*, *47*) and of protozoa (*38*) depends on the dose and age of inoculum. When age was kept constant, larger inocula have usually shown a shorter lag phase than smaller ones. Using the same dose for inoculation, organisms taken from older cultures needed a longer lag before they could start to grow than did members of younger cultures, at least when the increase of cell numbers and not of cell volume or mass was measured (*48*).

As with bacterial cultures, the latency period of the Ehrlich ascites tumor showed an inverse relationship to the dose of inoculum. Variation of the physiological age of the inoculum between 3 and 16 days did not show any certain effect on the length of the latency period, however. According to Hershey (*49*), the difference in length of lag phase between young and old bacteria is due to the fact that old cells are smaller than younger ones and require a longer time to reach the size at which division occurs. It was found in the present investigation that 3- and 16-day-old ascites tumor cells did not differ significantly in their average volume. If Hershey's interpretation is correct, and applicable to our cells, the variation of inoculum age between these limits could not be expected to show any effect on the length of the latency period, which is consistent with the experimental findings.

In analogy with Hinshelwood's (*47*) hypothesis for bacterial lag, the effect of different inoculum doses on the latency period of the Ehrlich ascites tumor could be interpreted by assuming that cell proliferation starts only after a definite critical concentration of some active intermediate has been built up within the peritoneal fluid, due to the activities of the inoculated cells. Considering the rapid circulation of peritoneal fluid (*50*) the number of cells inoculated should then be higher than a certain critical level in order to make it possible for the necessary substance to reach the critical concentration at all. This is in conformity with the previously demonstrated fact (*6*) that a certain critical number of cells

has to be inoculated in order to produce ascites tumors in all animals. No primary growth of free tumor cells can occur in the peritoneal fluid if the dose of inoculum is much smaller than the critical level, although the cells of such a small inoculum are usually able to produce solid tumors in the subcutaneous or retroperitoneal tissues of the host (often at the site of inoculation). After these solid tumors have grown to a large size, they may ulcerate to the peritoneal cavity and a secondary production of ascites may sometimes occur. The original small inoculum is, however, unable to survive and grow directly in the peritoneal fluid.

The better survival and growth of a small inoculum in the solid tissues than in the peritoneal fluid could be due to the less severe dilution and slower diffusion of the hypothetical active substance. Thus, a relatively small number of cells could be sufficient to build up the critical concentration necessary for cell division in solid tissues, whereas in the peritoneal fluid the products of such a few cells become rapidly diluted by the relatively large amount (about 0.12 ml.) of continuously circulating liquid.

Another parallel between the growth of ascites tumor cells and of microorganisms is that the final maximum number of free tumor cells in the peritoneal fluid is practically independent of inoculum size. The same has been found to be true for bacteria, yeasts, protozoa (*38*), and tissue culture cells (*44*). The size of the maximum population appears to be different for different ascites tumors; the MC1M ascites sarcoma ceased to grow when it reached about one-third the maximum size of the Ehrlich ascites tumor. This finding is also in agreement with the experience of the bacteriologists (*27*) that the final maximum number of cells varies with different bacterial species when the environmental conditions are kept constant.

Conclusions

The following points emerge as main conclusions with respect to ascites tumor growth:

1) Some mouse neoplasms are capable of growth in the form of free tumor cells or cell complexes in the peritoneal fluid (*1–7*). The cells multiply continuously in the fluid and their proliferation is followed by an increase of ascites volume. The relative and absolute concentration of tumor cells is high after a certain stage has been reached, and approaches a "nearly pure culture" (*3–7*). Relatively few available mouse neoplasms are able to grow in this form (*39*). High growth rate and/or high degree of anaplasia seem to be necessary but are not in themselves sufficient for the transformation of solid into ascites tumors. There is experimental evidence to favor the assumption that cells able to grow in the peritoneal fluid—and conceivably also in other body fluids—arise by some procedure related to spontaneous mutation after a prolonged series of transfer generations in certain rapidly growing tumors [(*39*) and unpublished data].

2) The number of viable ascites tumor cells inoculated must be higher than a certain critical level in order to produce ascites tumors in 100

percent of the inoculated animals (*6*). Doses below this level produce an increasing proportion of solid tumors only, without ascites. Much smaller numbers of tumor cells seem able to multiply in the solid tissues of the host than in the peritoneal fluid.

The following phenomena have been found to be characteristic for two typical ascites tumors, the Ehrlich carcinoma and the MC1M sarcoma, when the number of cells inoculated was higher than this critical level:

3) After inoculation the tumor cells intermix with the available peritoneal fluid (about 0.12 ml. per 20-gram mouse) and its content of non-tumor cells (about 4.8×10^6 per 20-gram mouse). The cells injected can be recovered quantitatively from the peritoneal fluid soon after inoculation, provided that their number is higher than about 10^6. With lower numbers the recovery of injected cells is not quantitative. This may be due to the comparatively larger losses during the collection procedure, or to a disappearance of cells by adherence to the peritoneal surface, lysis, etc.

4) The length of the latency period (the time elapsing before the number of free tumor cells in the peritoneal fluid increases above the level of the inoculum) is inversely related to the inoculum dose, but is not influenced by varying the physiological age of the inoculum between 3 and 16 days.

5) After the latency period has ended, the number of tumor cells increases approximately linearly with the third power of time (cube-root phase, the cube roots of tumor-cell numbers fall close to a straight line). It follows that the average generation time increases continuously and linearly during this period and the relative multiplication rate $\left(\frac{dN}{dt} \cdot \frac{1}{N}\right)$ decreases, at first very abruptly and later more slowly.

6) The number of free tumor cells in the entire peritoneal fluid of the mouse increases to a certain maximum value, which is about 1,500 million tumor cells for the Ehrlich and about 500 million for the MC1M ascites tumor. After Ehrlich ascites tumor cells have attained this level, their multiplication is slowed down very considerably ("terminal period"), and death of the host animal soon follows. With the MC1M sarcoma, the tumor-cell number remains stationary for a longer period and may show a decrease terminally.

7) During the growth of the Ehrlich ascites tumor the amount of the newly formed peritoneal fluid ($V - V_o$, where V is the total volume of the ascites at a given time, and V_o is the amount of peritoneal fluid present in the normal mouse) is directly proportional at any given time to the total number of free tumor cells present in the fluid [equation (13), text-figs. 6 and 8 V]. As the total number of tumor cells increases, their concentration per milliliter approaches an asymptote, and for larger cell numbers (when $V \gg V_o$) it is practically constant, showing only small random variations. Measuring the body weight of mice inoculated with the Ehrlich ascites tumor, Lettré (*2*) assumed that the increase of ascitic fluid is directly proportional to the increase in number of the tumor cells. The

direct proof obtained in the present investigation confirms this assumption. The same simple relationships do not hold for the MC1M tumor, however.

8) There are about 4.8 million free cells (n-cells) present in the peritoneal fluid of the normal, untreated C3H mouse with a body weight of about 20 grams. The number of n-cells increases during the growth of the ascites tumor. It could be shown that this increase is directly related to the multiplication of the free tumor cells. The relationship is adequately described by equation (14) for inoculum doses of the order of 10^6 cells. Equations (15) and (16) describe the percentage of tumor cells in the peritoneal fluid for any given total number of tumor cells. Previous observations that soon after inoculation the percentage of tumor cells is lower if a small number of cells was injected than if larger inocula were used, can be satisfactorily explained as a dilution of the injected tumor cells by n-cells, some of which pre-exist in the normal peritoneal fluid and some are contained in the inoculum. No major inflammatory reaction could be demonstrated for the two ascites tumors used when the dose of inoculum varied between 0.7 and 17.5 million cells (*cf.* text-figs. 7 and 8).

9) It was possible to confirm the previous conclusion (*5*, *7*) that solid tumors or infiltrations in the peritoneal cavity are not prerequisite for ascites tumor growth. The free tumor cells multiply in the peritoneal fluid first in the absence of, and later probably independently of the tumorous infiltration of solid tissues. For the Ehrlich ascites tumor it has been shown that such an infiltration is never detectable earlier than 4 to 5 days after inoculation, *i.e.* at a time when considerable multiplication of ascites tumor cells has already taken place.

10) The percentage of dead cells was negligible during the entire growth period of the Ehrlich ascites tumor. The same is true for the ascites form of the MC1M sarcoma except for the last stage, when the percentage of dead cells increased.

11) In agreement with previous observations by others on solid tumors (*43*, *24*) and tissue cultures [*ref. see* (*24*)], it has been demonstrated that the growth rate of the two ascites tumors investigated here does not exceed the embryonic growth rate of the mouse. (It should be noted that the Ehrlich ascites tumor is one of the most rapidly growing mouse neoplasms known.)

12) No significant difference could be demonstrated between the linear growth rate of: a) the original MC1M solid tumor, which has never been transferred to the peritoneal cavity; b) the MC1M solid tumor that developed from the subcutaneous inoculation of the MC1M ascites tumor; and c) the cube roots of the number of cells in the MC1M ascites tumor.

This general similarity between the growth curves of a tumor in its solid and ascites form suggests that at least for the MC1M sarcoma, the shape of solid-tumor growth curves is primarily determined by the course of tumor-cell multiplication *in vivo* and is relatively uninfluenced by central necrosis, intercellular variations with regard to nutritional conditions, etc.

13) There is no reason to assume that the multiplication of tumor cells *in vivo* proceeds at a constant rate according to the exponential law of growth during any major part of the growth period amenable to experimental study at the present time.

With regard to item 5, it should be kept in mind that a representation of the cube roots of tumor-cell numbers by a straight line during the "cube-root phase" of growth is only an approximation. Probably other types of equations could be fitted as well to shorter or longer parts of the ascites tumor growth curves. The cube-root relationship was selected for comparative and descriptive purposes only, and no attempt was made to formulate detailed theories on the metabolism and growth of the cells on the basis of this equation. It was chosen because there are only a few constants involved and all of them have a definite meaning. Also, the obvious direct relationship of the cube-root function to available growth curves of solid tumors, based on the fact that the mean diameter of certain transplanted solid tumors increases linearly with time (*20–24*), makes possible direct comparisons between the growth of solid and ascites tumors and also between neoplastic and normal embryonic growth.

The fact that mean generation time increases and relative growth rate decreases during the entire growth period is probably due to an increasing shortage of available nutrients per cell and per unit of time, due to the continuous multiplication of cells and the increase of fluid volume. Accumulation of metabolites, deterioration of the host animal, or other alterations in the host-tumor relationship can also play an important role. From the findings of Warburg and Hiepler (*11*) it would appear that oxygen and glucose may be among the major limiting factors. These authors found that anaerobic conditions prevail in the well developed Ehrlich ascites tumor, and that the ascites serum usually contains very little sugar. They have concluded that the oxygen and sugar consumption of the tumor cells does not depend on their metabolic capacity but on the relatively small amounts of oxygen and sugar available in the fluid. The shortage of these and other nutrients must increase greatly with increasing cell numbers. The mean distance of the average cell from the nutrient peritoneal surfaces becomes larger as the amount of fluid increases. This average distance, being a linear measure, is the cube-root function of fluid volume. But fluid volume is a direct function of cell number and therefore the distance is a function of the cube roots of tumor-cell numbers. It has been found [equation (9)] that mean generation time increases as a direct function of the cube root of the tumor-cell number. It might be speculated that the increase in mean generation time is related to the increasing mean distance from the nutrient surfaces of the average cell.

In the opinion of Warburg and Hiepler (*11*) it is surprising that the Ehrlich ascites tumor cells can survive at all in the peritoneal fluid in view of the small amounts of nutrients available in the advanced ascites condition, and they raise the question as to what amounts of energy are sufficient to keep the cells surviving *in vivo*. It has been found in the present investigation that the cells of the MC1M sarcoma cease to grow in the ascitic fluid at a much earlier stage (lower total number of tumor cells and smaller volume of fluid) than those of the Ehrlich tumor. It is possible that only exceptional strains of neoplastic cells can survive and grow under the nutritional conditions in the peritoneal fluid. Conversion of solid tumors into ascites tumors may depend on the occurrence of "mutants" with less exacting nutritional requirements than the majority of the original cell population (*cf.* conclusion 1 above).

Summary

The growth of two established ascites tumors, the Ehrlich carcinoma and MC1M sarcoma, has been studied by quantitative methods. Growth curves were obtained indicating the total number of free tumor cells in the entire peritoneal fluid of the mouse at different times after inoculation.

Inoculation was followed by a short period during which cell numbers remained stationary or decreased. This phase was termed the latency period and its length was found to vary inversely with the inoculum dose. Variation of the physiological age of inoculum between 3 and 16 days did not have any effect on the latency period of the Ehrlich ascites tumor.

The rate of tumor-cell multiplication was maximal shortly after the end of the latency period and decreased continuously thereafter. The initial maximum rate of growth was inversely related to the dose of inoculum, at least when cell numbers above the critical level needed for the production of ascites in 100 percent of the mice were used.

Cell multiplication never progressed at a constant rate according to the exponential law of growth. It was rather characterized by continuously increasing mean generation time. An approximately straight-line relationship was obtained during the major part of the growth period when the cube roots of tumor-cell numbers were plotted against time. This is a striking parallel to the well known fact that the diameter of certain solid transplantable tumors increases linearly with time, since the cube root of ascites cell numbers is comparable to the linear measure of a solid tumor. It is suggested that this type of solid-tumor growth is not an effect of central necrosis but depends on the general course of tumor-cell multiplication *in vivo*.

The period during which cell multiplication could be described by this straight-line relationship was referred to as the cube-root phase. There seems to be a definite optimum size of inoculum that produces the highest increase rate of the cube roots of tumor-cell numbers during this period.

Cell multiplication slowed down considerably (Ehrlich tumor) or stopped (MC1M) when a certain maximum number of free tumor cells was attained. This maximum number was independent of the size and physiological age of the inoculum, but was different for the two different neoplasms. The period following the end of the cube-root phase and preceding the death of the host was called the terminal period.

The increase in the number of non-tumor cells in the exudate is approximately directly proportional to the increase in the number of ascites tumor cells during the entire period of growth.

The increase in the amount of peritoneal fluid is directly proportional to the number of free tumor cells during the major part of the growth of the Ehrlich ascites tumor.

The great majority of free tumor cells are viable during the entire period of growth of the Ehrlich ascites tumor and, with the exception of the terminal period, of the MC1M sarcoma. The average volume of the tumor cells does not change with time to any detectable degree. The average length of mitosis is about one hour in the Ehrlich tumor.

The multiplication of free tumor cells in the ascitic fluid proceeds first in the absence of, and later probably independently from, the infiltration of tumor into solid host tissues. No infiltration was detectable earlier than 4 to 5 days after inoculation.

The Ehrlich ascites tumor closely approaches the growth rate of the mouse embryo but does not exceed it.

No significant difference was found between the growth rates of the MC1M sarcoma in its solid or ascites form.

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PLATE 21

FIGURES 1 and 2.—Ehrlich ascites tumor. Smears, prepared by the Papanicolaou method, of the ascitic fluid. × 1,200

FIGURES 3 and 4.—MC1M ascites sarcoma. Smears, prepared by the Papanicolaou method, of the ascitic fluid. × 1,200

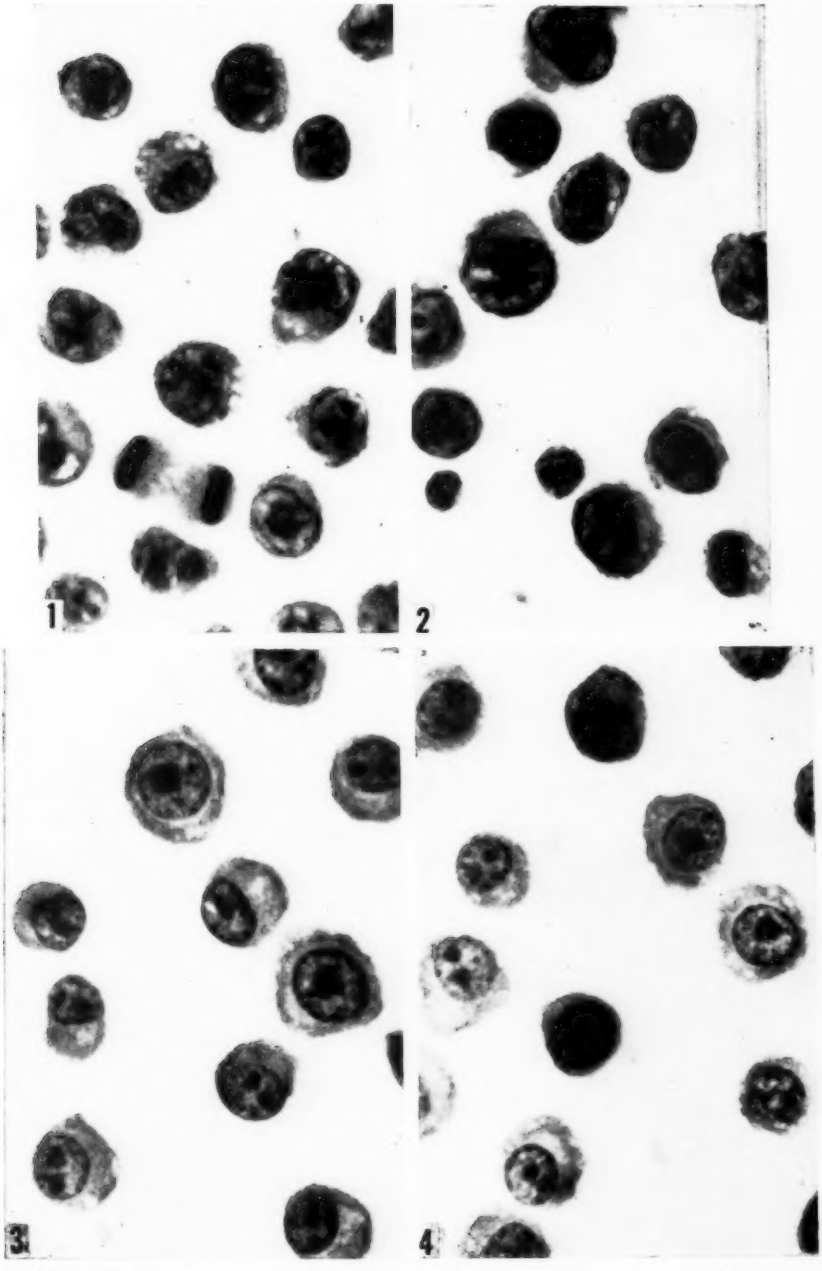

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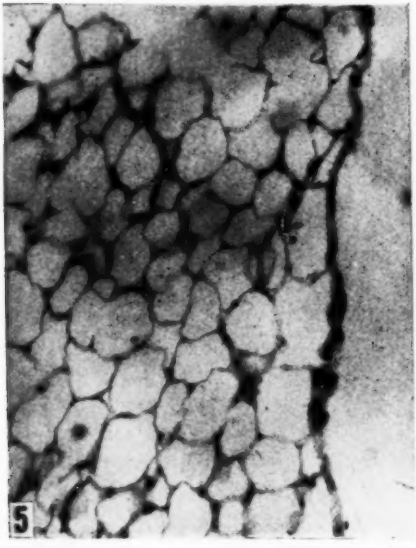
PLATE 22

FIGURE 5.—Omentum of a mouse 3 days after the inoculation of 1.8 million Ehrlich ascites tumor cells. No invasion of tumor cells visible. Hematoxylin and eosin. × 300

FIGURE 6.—Same as figure 5, 4 days after inoculation. A few tumor cells are attached to the peritoneal surface. No infiltration.

FIGURE 7.—Same as figure 5, 5 days after inoculation. Beginning of tumorous infiltration.

FIGURE 8.—Same as figure 5, 7 days after inoculation. Advanced tumorous infiltration.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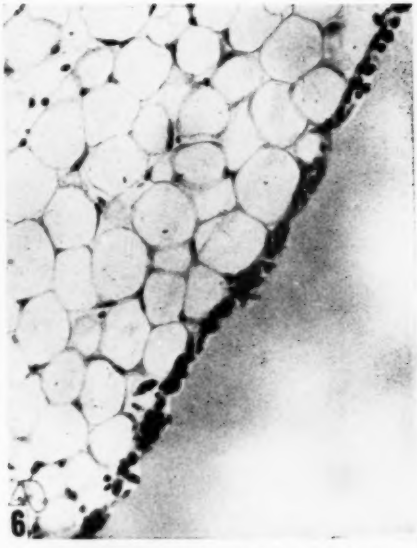
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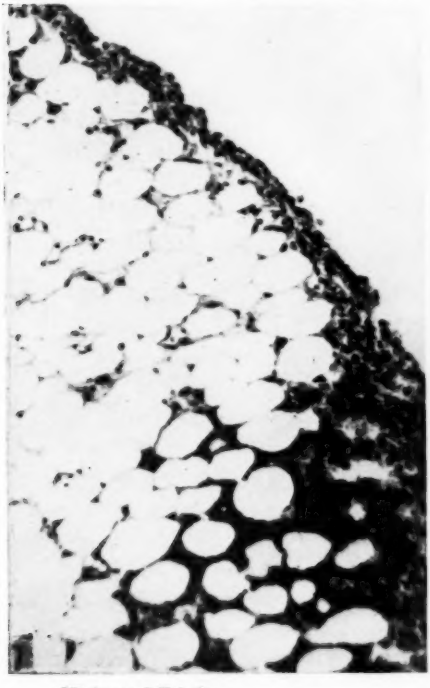
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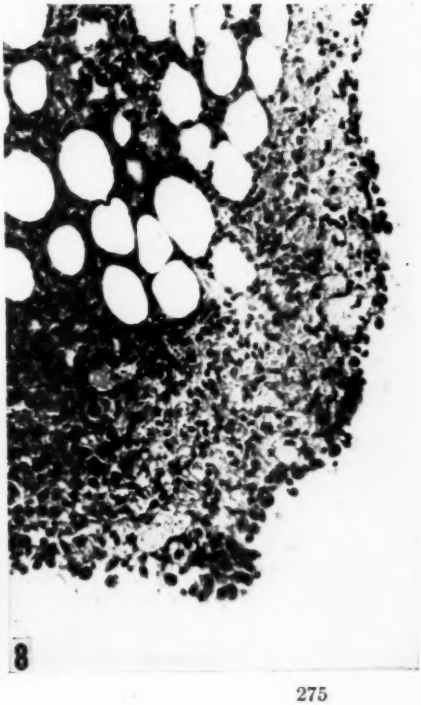
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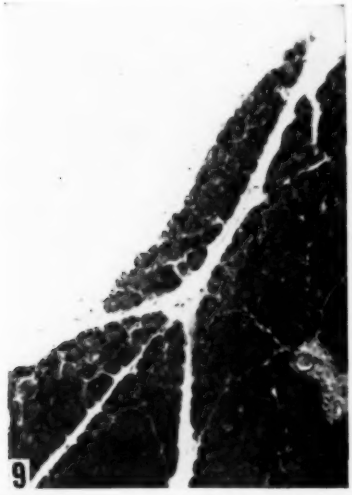
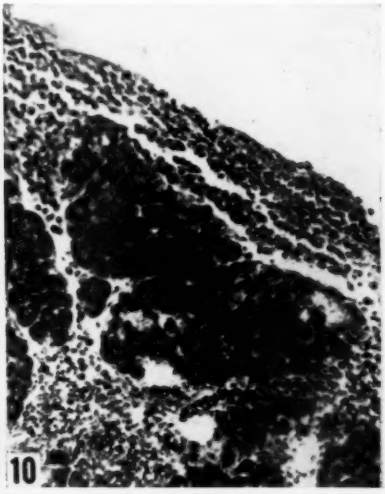
PLATE 23

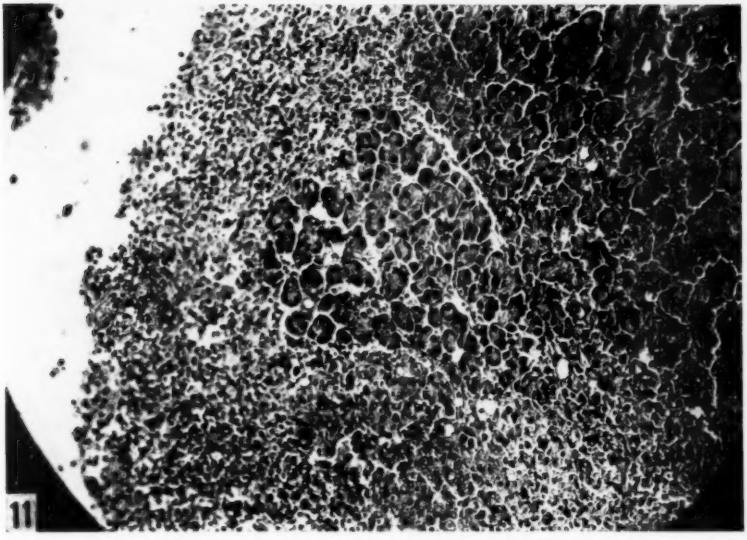
FIGURE 9.—Pancreas 4 days after the inoculation of 1.8 million Ehrlich ascites tumor cells. No invasion of tumor cells visible. Hematoxylin and eosin. × 100

FIGURE 10.—Same as figure 9, 6 days after inoculation. Beginning of tumorous infiltration.

FIGURE 11.—Same as figure 9, 9 days after inoculation. Pronounced tumorous infiltration.







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A Method for the Quantitative Spectrophotometric Measurement of 7-Hydroxy-2-Acetylaminofluorene [1]

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It has been established by several investigators that 7-hydroxy-2-acetylaminofluorene (7-OH-AAF) is one of the metabolites of the carcinogen 2-acetylaminofluorene (AAF), and it is therefore a probable metabolite of 2-aminofluorene (AF), also. Bielschowsky (*1*) isolated 7-OH-AAF from the urine of rats following oral administration of AAF and was able to account for 5 to 8 percent of the administered carcinogen in the hydroxylated form. Using paper chromatograms and isotope dilution methods, Weisburger *et al.* (*2*) identified 7-OH-AAF in the urine of rats ingesting 2-acetylaminofluorene-9-C^{14}. In view of these results, a specific color test for 7-OH-AAF would be of great advantage in studying the metabolism of AF and AAF.

The color test developed by Westfall and Morris (*3*) for the spectrophotometric estimation of AAF consists of diazotizing the hydrolyzed compound and coupling it with R-salt (sodium 2-naphthol-3,6-disulfonate). Under the conditions of the test, 7-OH-AAF also gives a color with very nearly the same absorption maximum as that of the AAF derivative. This fact was reported by Gutmann (*4*) and confirmed in this laboratory. Therefore the test cannot be used to determine amounts of 7-OH-AAF in the presence of AAF or vice versa.

In studying the diazotization of pure solutions of hydrolyzed 7-OH-AAF, a bright yellow color was observed immediately after the addition of sodium nitrite solution. No such yellow color appeared in solutions of hydrolyzed AAF, indicating that this color test could be used to differentiate the hydroxylated derivative from the parent compound. Studies were carried out, as described below, to determine the optimum conditions for the quantitative estimation of 7-OH-AAF and some preliminary work was done on the application of the method to animal tissues and excreta.

Development of the Method

Standard solutions of pure 7-OH-AAF [4] in glacial acetic acid were made,

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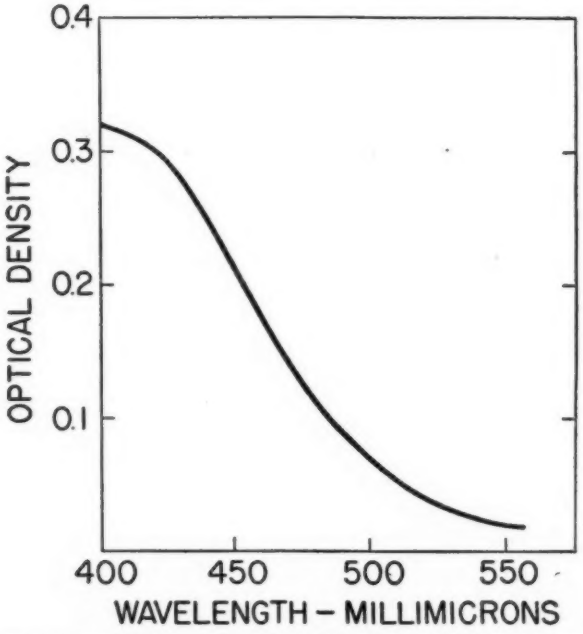
[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[4] This compound was prepared by Dr. J. H. Weisburger in this laboratory and melted at 232–233° C. uncorr.

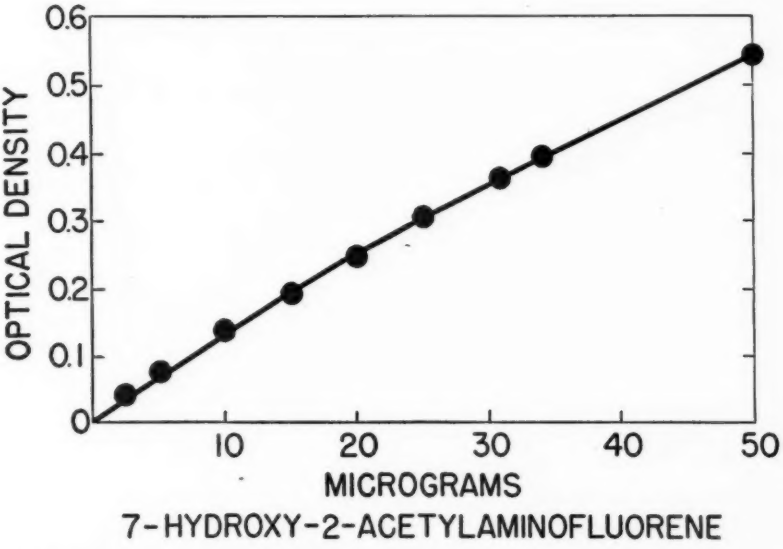
such that 0.5 ml. of the acid contained the desired amount of the compound. This quantity of the acetic acid solution was then mixed with 0.25 ml. of concentrated HCl and 2 ml. of distilled water in a glass-stoppered graduated 15-ml. centrifuge tube, and the tubes were immersed in a boiling water bath for one hour. They were then removed and cooled to 0° C. in an ice bath. One ml. of 0.03 M sodium nitrite was added to each of the experimental tubes and the volume in the tubes was then adjusted to 6 ml. by the addition of distilled water. The volume in the blank tubes was adjusted to 6 ml. with distilled water but no sodium nitrite solution was added. The color was read 15 to 30 minutes after nitrite addition, in a Coleman Spectrophotometer Model 11, using cuvettes of 16.5 mm. inside



TEXT-FIGURE 1.—Spectrophotometric absorption curve of the compound produced by the addition of 0.03 M $NaNO_2$ to 7-hydroxy-2-acetylaminofluorene that has been hydrolyzed in 1 N HCl.

diameter. Although the absorption maximum is close to 400 mμ (see text-fig. 1), the wave length chosen for reading was 450 mμ because the nitrite solution apparently absorbs light of wave lengths shorter than 430 mμ and this is not compensated by the blank tubes. Standard calibration curves, therefore, do not pass through the origin at these shorter wave lengths.

The color was found to follow Beer's law in the range of 0–25 μg. (text-fig. 2). Above 25 μg. the color does not increase as rapidly as it should for the concentration increase. Best results have been obtained when the sample tube contained between 10 and 20 micrograms.



TEXT-FIGURE 2.—Typical calibration curve for 7-hydroxy-2-acetylaminofluorene.

Since 7-OH-AAF will give the color with sodium nitrite only after the acetyl group has been removed by heating in the presence of HCl, it is possible to determine the amount of 7-hydroxy-2-aminofluorene (7-OH-AF) in a given solution by carrying out the test procedure with no prior heating. However, 7-OH-AF is more unstable, both in the dry state and in solution, than 7-OH-AAF and therefore was not used as a standard. Since the calibration curve was made from 7-OH-AAF, all values read from it would be expressed in terms of this compound but could, of course, easily be converted to weights of the free amine.

The specificity of the color was next tested, using as many derivatives of fluorene as were available, and three derivatives of the closely related biphenyl, which would be formed from fluorene were the methylene bridge at carbon 9 to be removed. At the same time, the amount of color given by these materials when coupled with R-salt was determined at 525 mμ and calculated as the percent of the color intensity from an equimolar amount of AF chromogen. The results for amounts of the test materials showing the greatest interference are given in table 1. Negligible interference was found with 2-monomethylaminofluorene, 2-dimethylaminofluorene, 1-diacetylaminofluorene, 4-acetylamino-4′-fluorobiphenyl, and *p*-acetylaminobiphenyl. Somewhat more interference was noted in the cases of AAF, AF, 2-diacetylaminofluorene, 2-acetylaminofluorenone, 2,7-diacetylaminofluorene, 1-hydroxy-2-aminofluorene,[5] and 3-hydroxy-2-aminofluorene.[5] None of these compounds except 2,7-diacetylaminofluorene and N,N′-diacetylbenzidine give a true yellow color in this test.

[5] These compounds were prepared by Dr. E. K. Weisburger in this laboratory.

The two *ortho*-hydroxy derivatives give a pinkish color with a maximum absorption at 470 mμ, and it is of interest to note that the hydroxyl group in the *ortho* position blocks the formation of the color with R-salt. The two compounds mentioned, which do give the characteristic yellow color, both have two *ortho-para* directing ring substituents in the same position relative to each other and to 7-OH-AAF, except that the benzidine derivative has no methylene bridge. The test, therefore, seems to be specific with respect to the type of substituent groups and to the positions in the ring, but since amination of an aromatic nucleus by animal tissues is unlikely whereas hydroxylation is common, the probability is high that any metabolic product of AAF giving the nitrite test would have a hydroxyl group in the seven position.

To test the accuracy of the method in determining amounts of AAF and 7-OH-AAF in the same solutions, mixtures of known amounts of these compounds were made, hydrolyzed, and diazotized. Of the 4 ml. of solution present in the centrifuge tube, 2 ml. were used for the coupling with R-salt (*5*). The remainder was diluted to 6 ml. and the nitrite color read at 450 mμ. The total recovery is obtained as follows: the micrograms of 7-OH-AAF are estimated from the 7-OH-AAF standard curve for the nitrite method. The value corresponding to the galvanometer deflections at 525 mμ for the R-salt-coupled compounds is read from the AAF standard curve for this reaction. Since a given weight of 7-OH-AAF has been found, under our test conditions, to produce half the color intensity, when coupled with R-salt, of an equal weight of AAF, the value read from the AAF curve does not represent the true total when 7-OH-AAF is present. It may easily be corrected by adding to the total estimated from the AAF curve one-half of the value for 7-OH-AAF that has previously been read from the nitrite curve. The results of a series of such tests of the method are given in table 2. They show that the calculated recovery of 7-OH-AAF is likely to be high, especially when the ratio of AAF to 7-OH-AAF is as great as 8 to 1. The inaccuracy is due probably to the interference of AAF, which can contribute a color in the nitrite test equivalent to 10 percent on a molar basis (see table 1). This, therefore, makes the AAF recovery low, since its value is obtained by difference, but the two errors tend to cancel each other and the total recovery is close to the theoretical amount. This is of practical interest, since one might be able to ascertain with some accuracy the total amount of these two forms present, for instance, as metabolites in urine, although the determination of either one individually might be subject to 10 percent error.

Recovery studies of 7-OH-AAF added to urine verified an experimental observation that the urinary pigment must be suitably diluted out before a 100 percent recovery is obtained. This dilution of urine has also been found necessary in the case of the color produced by coupling with R-salt. The darkening of pigment produced during the boiling for one hour in 1 N hydrochloric acid may prevent an accurate reading of the experimental color. This interference is especially noticeable in acidified urine containing spilled food. It is advisable to run the test at several different dilutions

TABLE 1.—*Interfering colors produced by fluorene derivatives and related compounds in the determination of 7-OH-AAF and of AAF*

Compound	μmoles present	7-OH-AAF determination		AAF determination		
		μmoles calculated as 7-OH-AAF	Percent interference	μmoles calculated as AAF	Percent interference	Wave length of maximum absorption, mμ
AAF	0.28	0.029	10.4	.....	.....	525
2-aminofluorene	.18	.018	10.0	.....	.....	525
2-monomethylaminofluorene	.35	(*)	.....	0.014	4.0	525
2-dimethylaminofluorene	.33	.006	1.8	.014	4.2	470
2-diacetylaminofluorene	.24	.026	10.8	.....	.....	525
1-diacetylaminofluorene	.24	.010	4.4	.11	47	505
2,7-diacetylaminofluorene	.11	.021	18.7	.11	100	560
1-hydroxy-2-aminofluorene	.21	.019	9.1	0	0	.....
3-hydroxy-2-aminofluorene	.05	.009	18.0	0	0	.....
2-nitrofluorene	1.24	(†)	.....	.....	.....	.....
2-acetylaminofluorenone	.21	.021	10.0	.189	90	515
4-acetylamino-4′-fluorobiphenyl	.22	.004	1.7	.187	85	515
p-acetylaminobiphenyl	.22	.002	1.0	.200	91	515
N,N′-diacetylbenzidine	.22	.020	8.9	.424	193	550

* Turbid, no color.
† Precipitate, no color.

TABLE 2.—*Recoveries of AAF and 7-OH-AAF from mixtures of the two*

Amounts added		Percent recovered		Percent of AAF + 7-OH-AAF recovered*
AAF γ	7-OH-AAF γ	AAF	7-OH-AAF	
13. 5	24. 1	92	112	102
25. 0	29. 0	88	110	100
26. 3	26. 8	89	112	100
25. 0	14. 2	90	112	98
46. 8	6. 37	91	136	97
47. 8	6. 37	92	152	100

*$\left[\begin{matrix}\text{Percent of AAF} \\ \text{+7-OH-AAF recovered}\end{matrix}\right] = \left[\frac{\mu\text{g. read as AAF} + 0.5 \times \mu\text{g. 7-OH-AAF found} \times 100}{\mu\text{g. AAF added} + \mu\text{g. 7-OH-AAF added}}\right]$

to see that maximum recoveries are obtained and that pigment of the higher concentration is not masking the test color. An example is given in table 3, where different amounts of 7-OH-AAF were added to varying dilutions of a urine that had darkened considerably on standing. In this case, in order to obtain a recovery consistently close to 100 percent, it was necessary to dilute the urine with seven parts of water. The amount of dilution necessary when testing any given urine depends thus upon the ratio of pigment concentration to concentration of 7-OH-AAF. If the ratio is very high, a low recovery will be obtained. Unfortunately this cannot be remedied by adsorbing the pigments on Norit, for all the metabolites are adsorbed as well.

TABLE 3.—*Recoveries of 7-OH-AAF added to rat urine diluted with varying amounts of distilled water*

Dilution of urine	μg. 7-OH-AAF present	μg. 7-OH-AAF found	Percent recovered
1:1	31. 6	23. 3	74
1:1	21. 6	13. 1	61
1:1	11. 6	8. 5	73
1:3	27. 8	22. 3	80
1:3	24. 5	20. 5	84
1:3	18. 3	15. 0	82
1:3	11. 0	9. 5	86
1:7	20. 0	19. 9	99. 5
1:7	20. 0	18. 7	93. 5
1:7	12. 5	13. 0	104
1:7	12. 5	12. 5	100
1:7	5. 7	5. 5	96. 5

Studies were made of the effect of temperature, of nitrite concentration, of hydrogen ion concentration, and of time on the amount of color produced, in an effort to characterize the color-producing reaction more closely. The results are summarized below.

Temperature

When the tubes containing the hydrolyzed material were kept at a temperature of 90° C. during the addition of sodium nitrite solution, an

average of 13 percent less color was developed than when the nitrite was added in an ice bath. A set of standards at room temperature gave 9 percent less color than the duplicates in the ice bath. Low temperatures seem to favor the reaction slightly, but this is not because high temperatures interfere with the diazotization. Twenty percent more color is produced in the R-salt coupling of hydrolyzed 7-OH-AAF when the diazotization is done at 90° C. than when it is carried out in an ice bath. Since the R-salt coupling of 7-OH-AF produces only about half the color intensity of the R-salt coupling of AF, it is suggested that the nitrite color may be produced by a self-coupling of two diazotized 7-OH-AF molecules, leaving only one diazotized position out of each two molecules free to couple with the R-salt. The effect of increased temperature might then be explained by assuming that it favors a dissociation of the self-coupled compound, freeing more diazotized amino groups to couple with the R-salt. There is as yet, however, no direct evidence to support this hypothesis.

Nitrite Concentration

The standard curve was made using a concentration of 0.03 M $NaNO_2$ because this was the concentration chosen by Westfall and Morris for the diazotization of hydrolyzed AAF prior to coupling it with R-salt. The use of this concentration thus makes possible the estimation of AAF and 7-OH-AAF in aliquots from the same hydrolyzed and diazotized mixture. Reducing the concentration to 0.015 M $NaNO_2$ causes a 20 percent increase in the amount of color produced by a given weight of 7-OH-AAF. This lower concentration has been found to produce the maximum color in standard solutions of 7-OH-AAF, but in urine, where nitrite is rapidly destroyed by urea, it has been found advisable to use the 0.03 M $NaNO_2$, and this is the concentration routinely employed.

Hydrogen Ion Concentration

The color has been found to increase with decreasing *p*H over the range studied, *p*H 5 to *p*H .05. For complete hydrolysis of AAF in one hour in a boiling water bath, 1 N HCl was found necessary (*3*). Although 2 N HCl produces more color in the nitrite test, it could also cause more destruction and greater charring during the hydrolysis. The use of 1 N HCl has therefore been adopted in this method.

Time

In pure solutions of hydrolyzed 7-OH-AAF the color produced by the addition of nitrite decreases from an initial maximum to a value that is fairly stable during the period of 15 minutes to one hour after nitrite addition. However, in urines the color sometimes fades more rapidly, probably due to the destruction of nitrite by urea. Therefore it is advisable to read such solutions in 15 to 30 minutes.

Application of the Method

The color test, hereafter designated as the nitrite test, has been applied to bile, tissues, and excreta of rats given AAF. This was done in order to determine whether compounds giving this color reaction are metabolites of AAF, and if so, in which tissues they are primarily concentrated.

For the studies on bile, fistulas were made by introducing polyethylene tubing into the common bile duct of rats under ether anesthesia, as described in another paper (*6*). These rats were each given, by stomach tube, single doses of 10 mg. per 100 grams of body weight of AAF labeled with N^{15}, and bile and urine were collected during the period that the animals survived. The amount of AAF recovered in bile and in urine was calculated both by recovered N^{15} and by the diazotization method (*5*). In no case was the recovery by the latter method more than 68 percent of the AAF calculated on the basis of the isotope present. Table 4 shows that in seven out of eight bile collections, and in five out of six urine samples collected, there was present a considerable amount of material giving the nitrite test. In all except the urine sample from Rat 33, this calculated 7-OH-AAF was significantly greater than that which could be accounted for as interference by AAF, assuming that all the diazotizable material was AAF.

TABLE 4.—*Application of the nitrite test to bile and urine of bile-fistula rats after a single gastric intubation of AAF-N^{15}* *

No. of rat	Period of collection	Material present, calculated as percent of AAF found by N^{15} determination					
		Bile			Urine		
		Diazotizable, uncorrected for 7-OH-AAF	7-OH-AAF † found by nitrite test	Diazotizable, corrected for 7-OH-AAF ‡	Diazotizable, uncorrected for 7-OH-AAF	7-OH-AAF found by nitrite test	Diazotizable, corrected for 7-OH-AAF
	(*hours*)	(*percent*)	(*percent*)	(*percent*)	(*percent*)	(*percent*)	(*percent*)
33	0–17	55	28	69	71	10	76
34	0–16.5	56	26	69	.....	.....	.....
	16.5–40	55	38	74	56	28	70
35	0–22	53	30	68	40	26	53
	22–46	0	0	0	50	0	50
36	0–17	63	24	75	48	18	57
	17–25	68	32	84	.....	.....	.....
	25–36	51	70	86	62	22	73

*See reference 6, table 4.

†Calculated on the assumption that the material giving this color test was all 7-OH-AAF.

‡All corrections are made by adding one-half of the value for 7-OH-AAF to the uncorrected diazotizable value as described for table 2 of this paper.

In order to obtain large amounts of the metabolites of AAF, eleven rats were given repeated doses of 10 mg. per 100 grams of body weight of AAF-N^{15}, three times weekly for a period of four weeks. Urine and feces were collected daily during this period. The excreta from Rat 1 CD were analyzed separately; those from Rats 2-11 CD were pooled. Both

the urines and the acetone extracts of the feces were found to contain considerable quantities of material giving the nitrite test, as shown in table 5. In the pooled urines of Rats 2-11 CD, one-sixth of the total AAF administered was excreted as 7-OH-AAF [6] or some acid-hydrolyzable derivative thereof, such as a glucuronide or ethereal sulfate. This is a great deal more than was found in urine by Bielschowski (*1*), who isolated 5 to 8 percent of administered AAF as 7-OH-AAF, but since our color test would presumably be given by glucuronides and sulfates of 7-OH-AAF after acid hydrolysis, this could account for the discrepancy. Moreover, information as to the total amount of hydroxylation taking place may be of more fundamental importance than knowledge of whether the resulting compound has a free or conjugated hydroxyl group. At any rate, the use of the nitrite test has led us to suspect that more of the aromatic nucleus of AAF undergoes hydroxylation than was hitherto supposed.

TABLE 5.—*Recoveries from rats given AAF-N^{15} by repeated gastric intubations*

Material	Percent of administered AAF		
	AAF calculated by N^{15}	7-OH-AAF found by nitrite test	Total diazotizable corrected for 7-OH-AAF
Urine, Rat 1 CD	46.7	8.9	30.0
Pooled urines, Rats 2–11 CD	49.2	16.5	41.3
92% acetone extract, feces, Rats 2–11 CD	13.8	3.9	10.4

A third group of rats was given nonisotopic AAF by stomach tube, killed within 2 to 4 hours, and certain of the tissues were homogenized and extracted with 92 percent acetone. The acetone extracts were evaporated to dryness in a current of air and the residues taken up in glacial acetic acid. This acetic acid solution was used in the nitrite test. Table 6 gives the results. In most of these tissues the large amount of fat present in the acetone extracts caused the final aqueous dilutions to be turbid

TABLE 6.—*Tissue contents of 7-OH-AAF several hours after administration of AAF*

Tissue	Micrograms of 7-OH-AAF per 100 grams of tissue		
	Pooled rats 12 + 13*	Rat 14†	Pooled rats 15 + 16‡
Liver	0	538	440
Kidneys + bladder + urine	1, 740	4, 300	432
Small intestine	4, 540	4, 300	.....
Large intestine	765	.....	.....
Skin	0	.....	.....
Muscle + bone	0	0	.....

* Killed 2 hours after gastric intubation of 10 mg. AAF per 100 grams of body weight.
† Killed 2½ hours after slow infusion of 2.5 mg. AAF per 100 grams of body weight, by tail vein.
‡ Killed 4 hours after gastric intubation of 10 mg. AAF per 100 grams of body weight.

[6] Hereafter the material giving the nitrite test will be called 7-OH-AAF.

and necessitated filtering before they could be read on the spectrophotometer. It is possible that some 7-OH-AAF may have been dissolved in the fat and thus lost, but the likelihood is not very great since the nitrite test carried out on several of these fatty residues after saponification gave no more color than did similar residues unsaponified.

It had been expected that the liver might show a fairly large concentration of 7-OH-AAF since the material had been found in bile (table 4). Actually, however, the material was either absent, or present in low concentrations, in extracts of liver at a time when a relatively large amount was present in the small intestine. This concentration of the material in the small intestine is found even when the AAF is given intravenously, as in the case of Rat 14, and is further evidence that bile is a major pathway of excretion. It is probable that the concentration of 7-OH-AAF in the liver at any one instant is small and it is rapidly carried via the bile to the small intestines, whence it is partially reabsorbed, to be excreted by the kidneys, and the remainder is excreted in the feces.

Summary

A quantitative spectrophotometric method has been described for the determination of 7-hydroxy-2-acetylaminofluorene (7-OH-AAF). The method is based upon the yellow color developed when a dilute solution of sodium nitrite is added to an acidic solution of 7-hydroxy-2-aminofluorene produced by acid hydrolysis of 7-OH-AAF. Compounds which give, on a molar basis, up to 10 percent of the color given by 7-OH-AAF include 2-acetylaminofluorene (AAF), 2-aminofluorene, 2-acetylaminofluorenone, 2-diacetylaminofluorene, 2,7-diacetylaminofluorene and N,N′-diacetylbenzidine. The measurement of 7-OH-AAF in the presence of AAF, of which it is a metabolite, results, therefore, in an overestimation of the 7-OH-AAF amounting to about 10 percent. Recoveries of 100 percent are obtained, however, when the sum of 7-OH-AAF and AAF is determined by measuring, in a given solution, both the nitrite color and the color after coupling with R-salt.

A positive nitrite test was obtained in acetone extracts of liver, kidney, and of small and large intestines within a period of 2 to 4 hours after administration of AAF. Moreover a significant amount of material giving the nitrite test has been found in the bile and urine of bile-fistula rats and in the urine and feces of intact rats, after these animals received AAF by stomach tube. These findings show that part of the difference between the amounts of diazotizable material and of N^{15} recovered from the rat after the administration of AAF-N^{15} can be accounted for by the presence of a considerable quantity of 7-OH-AAF.

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Prevention of Irradiation-Induced Lymphoid Tumors in C57BL Mice by Spleen Protection [1, 2]

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In their experiments on induction of lymphoid tumors in C57BL mice, Kaplan and Brown (*1*) observed that irradiation limited to one-half of the body failed to elicit tumors. Even when the volume dose in half-body irradiation was equal to the volume dose in total-body irradiation that induced lymphoid tumors in the majority of the animals, no increase in tumor incidence was found. Furthermore, in experiments in which one thigh was shielded with lead during whole-body irradiation, Kaplan and Brown observed a significantly decreased leukemogenic effect of the irradiation. The conclusion was reached from these experiments that the induction of tumors of this type cannot be entirely due to direct effects of the irradiation.

Observation of the modification of irradiation injury by spleen protection during irradiation (*2*) or by homologous bone-marrow injections post-irradiation (*3*, *4*) suggested that prolonged depression of hematopoietic tissues may play an important role in the induction of lymphoid tumors. Either spleen protection or bone-marrow injections result in a rapid recovery of hematopoietic tissues of mice given a total-body exposure that would otherwise be lethal. In the experiments of Kaplan and Brown where the thigh was shielded, bone marrow was protected. This surviving bone marrow should have an effect on the recovery of the hematopoietic tissues similar to that of an injection of homologous bone marrow or spleen protection. In the following experiments, which were started in the middle of 1951, it will be shown that rapidly regenerating bone marrow produced by spleen protection will prevent the high incidence of irradiation-induced leukemia in C57BL mice.

Experimental Procedure

One hundred eighty-one male and female mice, approximately 2 months of age, from Kaplan's C57BL colony [4] were used in the experiments. They were divided into three groups of 61, 61, and 59 mice. Sexes were

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[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.
[4] We are greatly indebted to Dr. Henry Kaplan for supplying us with these mice.

distributed evenly in the three groups. Groups I and II were given 225 r total-body exposure [5] repeated at weekly intervals to a total dose of 900 r. The fractionated irradiation of the first two groups was chosen because, according to Kaplan and Brown (*5*), an interval of four to eight days gives a higher incidence and shorter latent period than a single exposure or exposures at daily fractionation to the same total dose.

Since the number of mice available was too small to use some of them as donors for postirradiation homologous bone-marrow injections for the acceleration of the recovery of the hematopoietic tissues of the hosts, protection by lead of the surgically exteriorized spleen was employed, as described by Jacobson *et al.* (*2*). Histologic studies have revealed that both procedures are identical in their protective effects in acute irradiation injury. Group I served as irradiated control group without spleen protection. In group II, the spleen was exteriorized and lead-protected during all four exposures. In group III, which received a single exposure of 900 r, the spleen was also exteriorized and protected. The exteriorization of the spleen necessitates cutting a small blood vessel leading to the tip of the spleen, which frequently caused infarction. The spleen was also exteriorized in group I during the first exposure only, without protection by lead, however.

Irradiation was started in groups I and II when the animals were 2 months of age; group III was exposed at approximately 2½ months, the midpoint of the fractionated treatment of the first two groups. The animals were maintained on Purina dog chow and water *ad libitum*, and housed in plastic cages under identical conditions. Autopsies were performed on all animals that died or were killed when moribund. The following organs and tissues were routinely taken: thymus, bone marrow, spleen, lymph nodes, gonads, liver, and adrenal glands. Animals that died within 2 months after the first exposure were not counted, since lymphoid tumors have not been observed during this period. Animals that survived to 300 days after the beginning of irradiation were killed at that time since, according to Kaplan and Brown (*5*), the tumor incidence attains a plateau at 300 days or earlier. No nonirradiated control animals were kept because Kaplan and Brown (*5*) had found that a consistent spontaneous lymphoid tumor incidence of 6 to 8 percent appeared in strain C57BL mice from their colony. Leukocyte and differential counts were taken on 10 representative animals from each of the three groups.

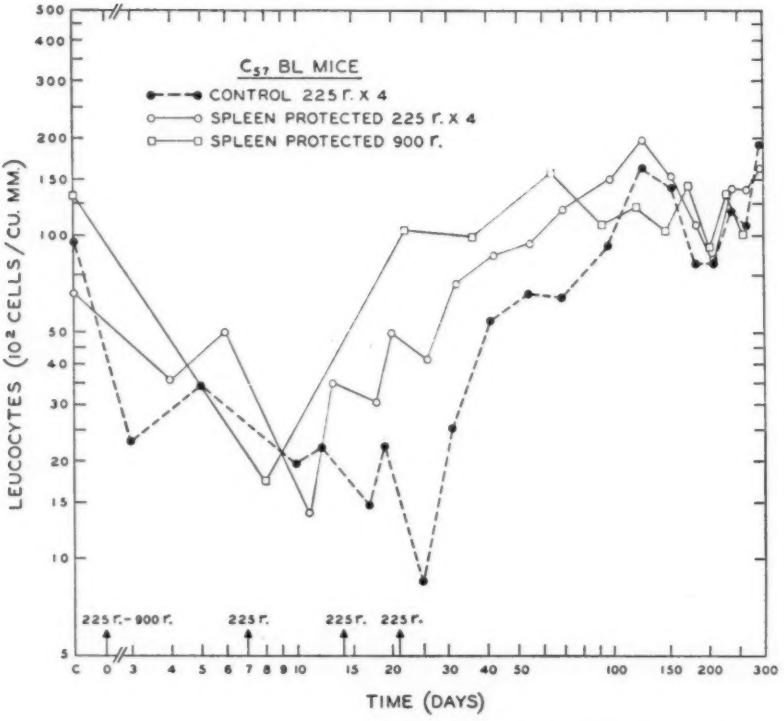
Data on lymphoid tumor incidence are presented in table 1. In group I, in which the spleen was not protected, a high incidence (68 percent) of irradiation-induced lymphoid tumors, with a mean tumor-development time of 198 days, was observed. In groups II and III, in which the spleens were protected, incidences of 2 and 0 percent, respectively, were found. The effect of the irradiations on the circulating leukocytes, heterophils, and lymphocytes is shown in text-figures 1–3.

[5] Physical factors of irradiation were: 2 tubes opposite each other; 186 KVP, 20 ma. each tube; beam filtration: 0.25 mm. Cu + 1.06 mm. Al, target-mouse distance for each tube 54 cm., dose rate 91 r per min.

TABLE 1.—*Incidence of lymphoid tumors in spleen-protected and non-spleen-protected irradiated C57BL mice*

Expressed as: $\frac{\text{Animals autopsied with lymphoid tumors}}{\text{Animals autopsied without lymphoid tumors}}$

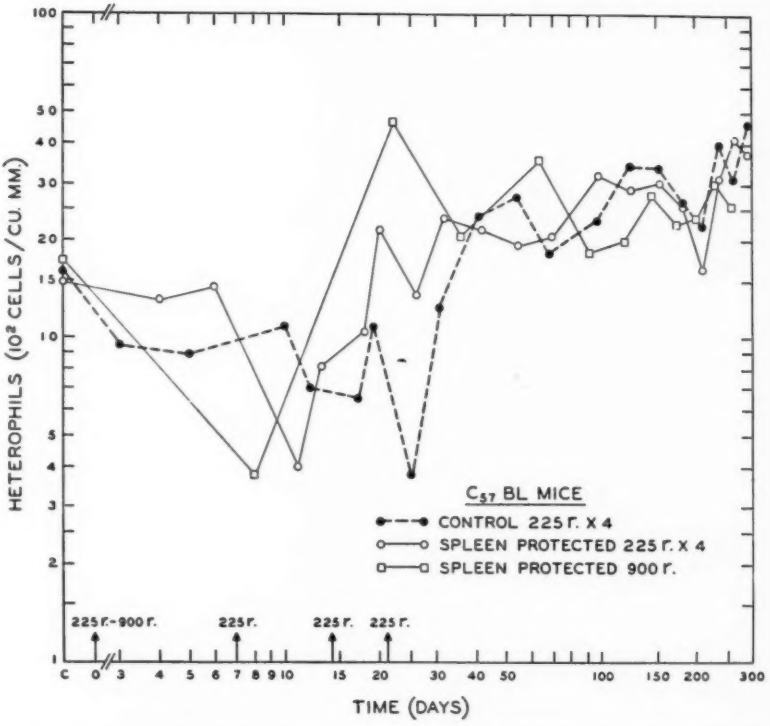
Exp. group	Dose (*r*)	Spleen protection	No. animals	No. surviving treatment	30-day intervals starting at 3rd interval after beginning of irradiation								Incidence (*percent*)
					3	*4*	*5*	*6*	*7*	*8*	*9*	*10*	
I.....	225 × 4.....	No.....	61	56	0/0	0/2	5/0	11/0	9/0	5/0	3/1	5/15	68
II....	225 × 4.....	Yes.....	61	44	0/0	0/0	0/0	0/0	1/0	0/2	0/0	0/41	2
III....	900.....	Yes.....	59	29	0/0	0/0	0/0	0/1	0/0	0/1	0/3	0/24	0



TEXT-FIGURE 1.—Circulating leukocytes in irradiated C57BL mice with and without spleen protection.

These text-figures indicate that the depression of the leukocyte count of the non-spleen-protected group is considerably more pronounced than in the spleen-protected groups, and that only after approximately 100 days were normal leukocyte values regained. In the second, and especially in the third group, the depression is less pronounced and the leukocyte values are normal at approximately 50 and 20 days, respectively, after irradiation. The prolonged depression of the leukocyte count is mainly due to a depression of lymphocytes (text-fig. 3), while the heterophils return rapidly to normal.

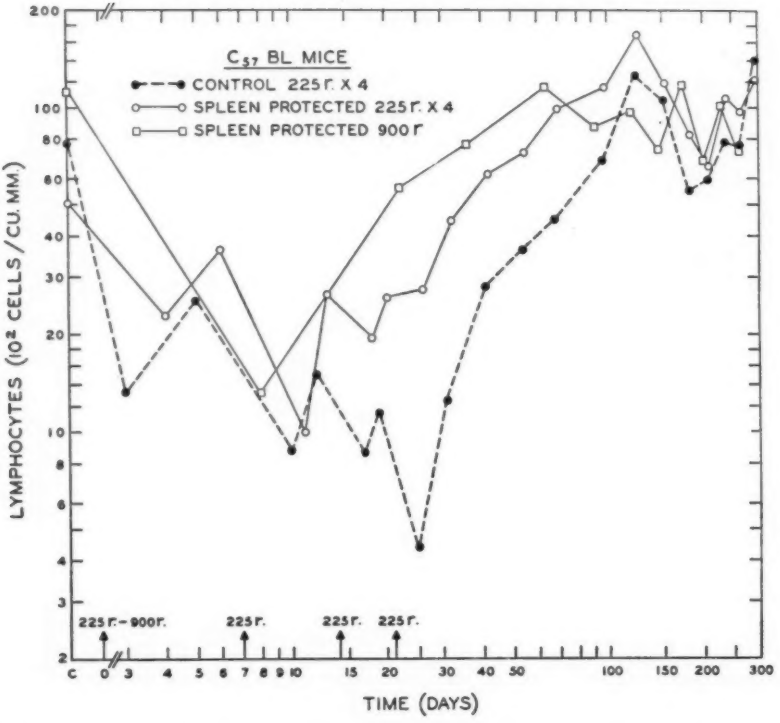
At autopsy, gross and microscopic examination of group I mice showed lymphoid tumors in a large percentage of animals. In the majority of cases the disease was widely disseminated with the thymic region usually showing a considerable tumor mass. In most of the mice this mass filled the upper part of the thoracic cavity, infiltrated the base of the heart and the lungs, and frequently extended along the anterior mediastinum to the diaphragm. Infiltration of the sternum adjacent to the thymus was usually observed. In rare instances the lymphoid tumor was largely limited to the thymus, causing an enlargement of one or both lobes.



TEXT-FIGURE 2.—Circulating heterophils in irradiated C57BL mice with and without spleen protection.

Spread of the tumor beyond the thorax was characterized by replacement of the normal tissue of lymph nodes, spleen, and Peyer's patches with neoplasm. Microscopically, infiltration of the bone marrow, liver, kidneys, adrenal glands, and gonads was regularly observed, but there was some variation in the number of tumor cells.

The 18 mice in this group that did not develop lymphoid tumors during the 300-day period of observation were particularly interesting because 4 showed a thymus that appeared histologically normal (fig. 1), while in 3 the thymus showed a great reduction in lymphocytes and the reticulum cells became very prominent, thus altering the normal architecture (fig. 2). These animals might well have developed lymphoid tumors if the period of observation had been extended, since atrophic changes of this type might possibly precede the development of some lymphatic neoplasms. A granulomatous lesion characterized by multinucleated giant cells occupied a large area of the thymus in one mouse. In 10 mice the thymus was not available for microscopic examination, but in these there was a record at autopsy of a small thymus, and the absence of neoplastic infiltration in any other tissue makes it reasonably certain that



TEXT-FIGURE 3.—Circulating lymphocytes in irradiated C57BL mice with and without spleen protection.

there had been no neoplastic change. Several of these 18 animals showed marked depletion of the sternal bone marrow (fig. 3) and partial depletion of bone marrow was frequent. In other respects they were similar to the mice in groups II and III in which only one lymphatic neoplasm was found. The single lymphoid tumor that appeared in the two spleen-protected groups did not differ anatomically from the tumors seen in group I. In the remaining mice all thymic tissue appeared normal on both gross and microscopic examination.

The sternal bone marrow in these two groups never showed cellular depletion, and granulocytopoiesis was marked (fig. 4). In the spleen, varying degrees of chronic productive perisplenitis and the formation of adhesions were noted. The proceeding operative procedures accounted for these changes. Many of the spleens appeared normal as far as the character of the red and white pulp was concerned. In others hyperplasia of the germinal centers was sometimes so extreme as to produce a nodular enlargement of the spleen. A similar change has been noted by Kaplan[6] in older nonirradiated C57BL mice.

[6] Personal communication from Dr. Henry Kaplan.

The lymph nodes in the two spleen protected groups, and in the few mice in group I that did not develop tumors, generally showed reactive changes that may be found long after irradiation, characterized by absence of germinal centers, reticuloendothelial hyperplasia, fibrosis, and mast cell increase.

Radiation damage was also observed in the ovaries and testes. Secondary changes in the adrenal glands associated with gonadal atrophy were extremely variable in amount. A few tumors of other types were observed in all three groups. The tumors were: a hemangioendothelioma of the skin, one ovarian tumor, one lung tumor, a hepatoma, an osteogenic sarcoma of the vertebral column, and a reticulum-cell sarcoma. These tumors were probably not induced by the irradiation.

Discussion

From the data given it is evident that spleen protection effectively inhibits induction of lymphoid tumors in C57BL mice by irradiation. The circulating lymphocytes showed a depression of long duration in group I. Histologic observation on mice of this group that came to autopsy at 300 days without lymphoid tumor development showed moderate to extreme depletion of bone marrow. In contrast, in the spleen-protected groups the lymphocyte count returned to normal earlier, and on histologic examination no reduction of cells was observed in the bone marrow. This indicates that prolonged bone-marrow depression and the concomitant prolonged reduction of lymphocytes in lymphoid structures may be a major factor in the induction of lymphoid tumors in C57BL mice.[7] The experiments of Kaplan in which he showed that irradiation of half the body or the shielding of a leg during irradiation prevented the development of lymphoid tumors may be explained on this basis. The reason that spleen protection or bone-marrow injections or bone-marrow shielding promote the rapid recovery of the irradiated bone marrow and lymphoid structures is still obscure. It is especially uncertain whether a humoral or cellular (repopulation) phenomenon is involved (*3*, *4*).

It can be proposed that the stress phenomenon caused by the surgical exteriorization of the spleen may have resulted in release of cortical hormones. Cortisone is known to have an inhibitory effect on the growth of established lymphoid tumors. However, Kaplan (*6*) has shown that injections of lipo-adrenal extract, 0.1 cc. intramuscularly 3 times a week for 2 weeks, had no significant effect on the incidence of irradiation-induced lymphoid tumors, although when 0.25 mg. of cortisone was given subcutaneously, two injections per week for 10 weeks starting at the time of the first treatment, lymphoid tumor incidence was reduced from 96 percent in untreated animals to 56 percent in the treated animals during the observation period of 270 days. Release of cortical hormones, there-

[7] This point of view was expressed in a preliminary note. Uphoff, D., and Lorenz, E.: Progress Report: Effects of spleen protection on lymphoid tumors induced in C57 black mice by total-body irradiation. Argonne National Laboratory. Quarterly Report, Nov., Dec. 1951 and Jan. 1952, Division of Biological and Medical Research, ANL-4745, 27-28, 1952.

fore, cannot account for the 2 percent and 0 percent tumor incidence in the spleen-shielded animals of groups II and III. The tumor incidence of 68 percent in the group in which the spleens were not shielded is lower than the incidence of 98 percent found by Kaplan and Brown (*5*) in experiments in which C57BL mice were irradiated four times to a total dose of 951 r with an interval of 8 days between exposures. This cannot be explained by the procedure of exteriorization of the spleens during the first exposure without shielding. However, at the first exposure our mice were 2 months of age, while Kaplan and Brown first exposed their mice at the age of 33 ± 3 days. Kaplan (*7*) had shown previously that lymphoid tumor incidence is an inverse function of the age of C57BL mice exposed under identical conditions. It seems, therefore, that the prevention of lymphoid tumor induction following spleen protection during irradiation is caused by an unknown factor in the bone marrow or in the spleen or in both, which stimulates the rapid recovery of lymphoid structures. Evidence is also presented that the induction of lymphoid tumors is dependent upon hypoplasia of bone marrow and lymphoid structures.

Summary

It has been shown that spleen protection effectively prevented the induction of lymphoid tumors in irradiated C57BL mice.

The circulating lymphocytes showed a considerable and prolonged depression in non-spleen-shielded irradiated mice in comparison to spleen-shielded irradiated animals.

The data indicated that prolonged depression of bone marrow and lymphoid tissue is an important factor in eliciting irradiation-induced tumors.

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(*6*) KAPLAN, H. S., MARDER, S. N., and BROWN, M. B.: Adrenal cortical function and radiation-induced lymphoid tumors in mice. Cancer Research 11: 629–633, 1951.

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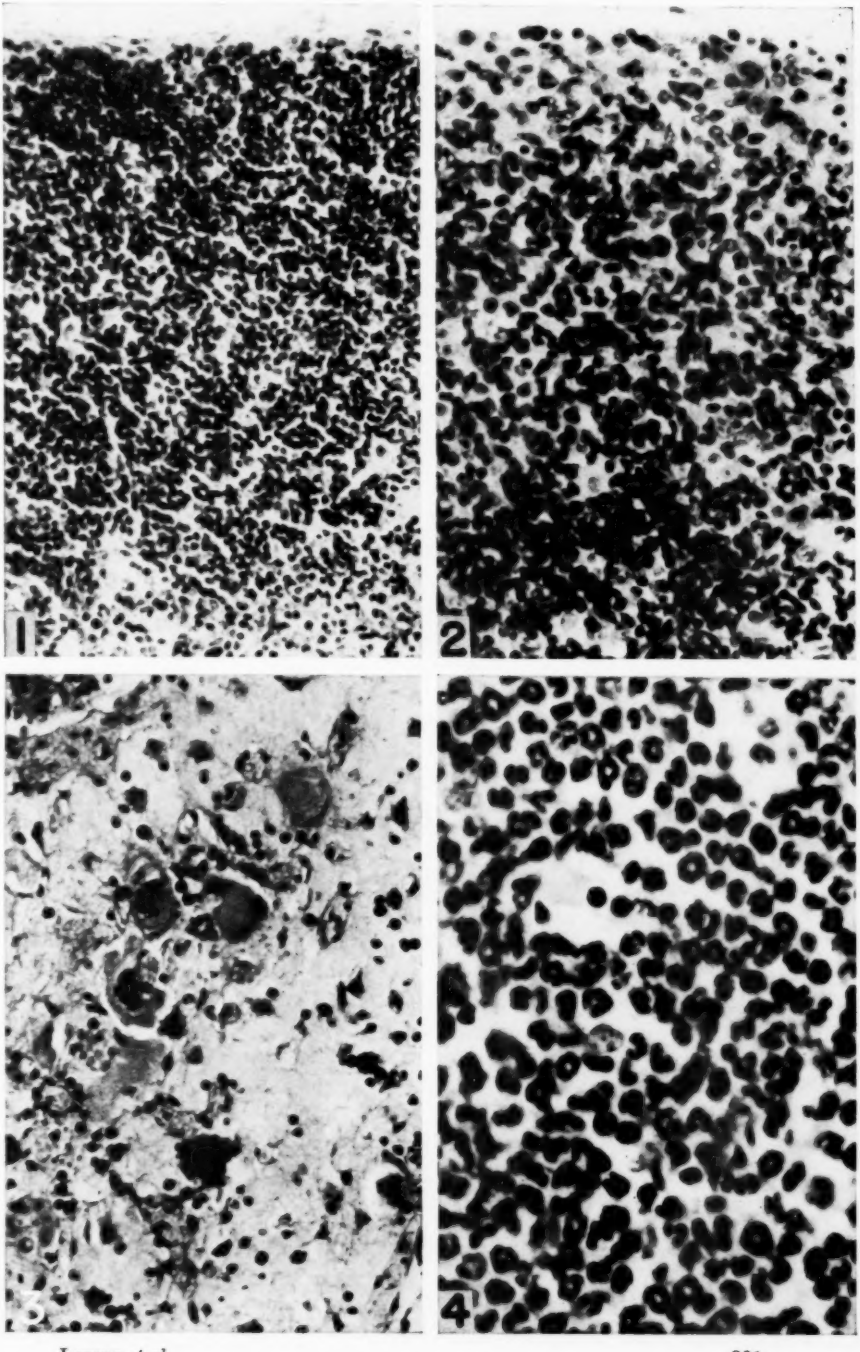
PLATE 24

FIGURE 1.—Thymus of a non-leukemic animal in the group without spleen protection, showing normal architecture of the cortex. Hematoxylin and eosin. × 290

FIGURE 2.—Thymus of a non-leukemic animal in the group without spleen protection, showing marked reduction in lymphocytes in the cortex. Hematoxylin and eosin. × 340

FIGURE 3.—Gelatinous sternal bone marrow in a non-leukemic animal of the group without spleen protection. Very few hematopoietic cells remain. Hematoxylin and eosin. × 320

FIGURE 4.—Abundant granulocytopoiesis in the sternal bone marrow of a mouse that was spleen-protected during the total-body irradiation. Hematoxylin and eosin. × 780



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Influence of Bone-Marrow Injections on Involution and Neoplasia of Mouse Thymus after Systemic Irradiation[1, 2, 3]

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Previous studies have established the paradox that C57BL mice, highly susceptible to lymphoid tumors of thymic origin after total-body irradiation, are almost completely refractory to their development after local irradiation over the thymic area (*1*). Moreover, the occurrence of such tumors may be largely prevented by shielding one thigh with lead during systemic irradiation (*2*). Thigh shielding has also been shown to promote regeneration of the thymus after acute radiation damage and involution (*3*). It has seemed reasonable to suppose that these two effects of thigh shielding upon the thymus are related, and that the mechanism that facilitates recovery from the initial radiation injury also acts to prevent lymphoid tumor genesis.

Recent radiobiologic investigations (*4–7*) have suggested that intact bone marrow might be the tissue responsible for these effects of thigh shielding. This possibility was reinforced by the observation that shielding of the tail (which usually contains only fatty marrow) offered no protection against lymphoma development (*8*). It has more recently been shown (*9*) that, although shielding of the intact rat tail does not reduce radiation mortality, shielding of rat tails rendered hematopoietically active by intra-abdominal implantation confers protection similar to that provided by spleen shielding (*4*) or bone-marrow injection (*6*) in the mouse.

Accordingly, a series of experiments has been performed to test the effects of injected bone-marrow cell suspensions on *a*) thymic regeneration, and *b*) lymphoid tumor incidence in irradiated C57BL mice. In a preliminary series of four experiments, reported elsewhere (*10*), it was found that cell suspensions of homologous bone marrow, administered intravenously after irradiation, were indeed effective in promoting thymic regeneration, though less effective in this regard than thigh shielding. The composite thymic weight curves of these experiments are reproduced

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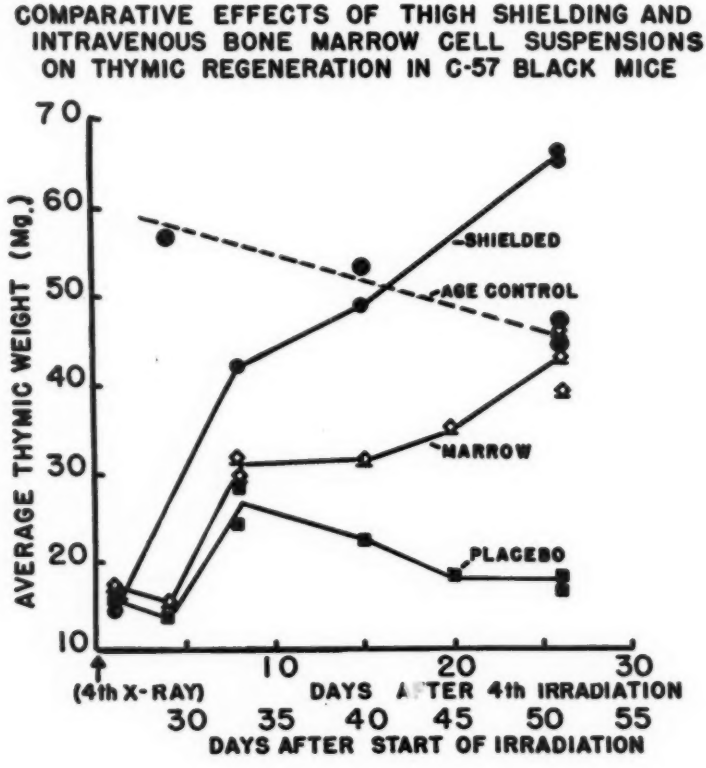
[2] Presented at the annual meeting of the American Association for Cancer Research, Inc., Chicago, April 9–11, 1953.

[3] These studies have been supported by grants from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, and from the Jane Coffin Childs Memorial Fund for Medical Research.

here for convenience (text-fig. 1). This paper reports additional experiments concerning the influence of bone-marrow injections upon both of the above-stated end points.



TEXT-FIGURE 1.

Materials and Methods

All experiments employed C57BL mice of both sexes, aged 33±3 days at the start of irradiation. Unless otherwise stated, litter mates were distributed at random among all groups of each experiment. Irradiated groups received a standard course of periodic total-body treatments: 4 doses of 168 r [4] each at 8-day intervals on experimental days 1, 9, 17, and 25. Bone-marrow cell suspensions were prepared in ice-cold Locke's solution by the method of Lorenz *et al.* (*6*). The suspended marrow from both femurs of each C57BL donor mouse (about one month old) constituted a single injection for each recipient animal, previous studies having indicated that smaller amounts of marrow are ineffective (*10*). Intravenous injections were made through a 27-gauge needle into the pre-

[4] Physical factors were: 120 KVP, 9 ma., 0.25 mm. Cu + 1.0 mm. Al added filter, 30 cm. target-mouse distance, output 32 r per min., HVL 0.39 mm. Cu.

warmed tail veins, and except where otherwise stated, were completed within one hour following X-ray treatment. The animals were then maintained without further treatment in identical cages with free access to Purina laboratory chow and water.

Experiment 1 was intended to compare the effects of intraperitoneally injected, preirradiated, and preshielded marrow with that of intravenous marrow on thymic recovery. Moreover, in the initital experiments cited (*10*), marrow suspensions had been injected after each of four X-ray doses. It seemed desirable to determine whether the same effect could be obtained with a single injection after the last irradiation.

Nine groups were set up and treated as indicated in table 1. Eight groups contained 4 females and 4 males each, with litters distributed at random. Group 9, added when the experiment had already been started, contained 3 female and 5 male mice. The placebo-treated animals (Group 1) received 0.25 ml. of Locke's solution intravenously after each irradiation. Normal marrow suspended in Locke's solution was injected after each X-ray dose, intravenously in Group 4, intraperitoneally in Group 3; and intravenously after the final X-ray dose only in Group 5. Group 2 received 4 intravenous injections of irradiated marrow taken from donor mice exposed to a single total-body dose of 450 r 48 hours before sacrifice. Group 6 was similarly injected with marrow from the lead-shielded femurs of mice exposed to 168 r 48 hours before sacrifice. Group 7 animals were irradiated on immobilization boards with a lead strip 1 cm. wide and 3 mm. thick covering one thigh; they received no marrow or other injections. Group 9 received intravenous injections of 0.5 ml. of normal whole blood from the same donor mice whose marrows were used for Groups 2 and 3; inadequate measures to prevent clotting resulted in embolic deaths, however, in 1 female and 3 males in this group. Group 8 remained untreated and served as an age control.

All groups were etherized on day 50, 25 days after the last X-ray treatment. The thymus, spleen, and four pooled superficial lymph nodes (2 inguinal, 2 axillary) were rapidly excised, dissected free of surrounding fat, and weighed on a torsion balance. They were preserved in Bouin's fluid and processed for histologic study, together with marrow from one femur. Smears were made of the marrow from the other femur and stained by Giemsa's method.

Experiment 2 was aimed at determining whether the thymus-regenerating marrow activity was a property of whole cells or a readily extractable material. Litter mates of both sexes were distributed among 6 groups, and an additional group of males for age control was segregated from a pool of marrow-donor mice of the same age. All but the age controls received four total-body irradiations. Within one hour after the last X-ray dose, the groups were intravenously injected, respectively, with Locke's solution; centrifuged supernate; marrow cells lysed in distilled water; centrifuged, washed, and suspended marrow cells; recombined cells and supernate; and whole bone-marrow suspension.

The bone marrow from both femurs of 30 donor mice was pooled and

suspended in cold Locke's solution in two calibrated 5-ml. centrifuge tubes, the volume in each being made to 3.75 ml. After stirring, 2.5 ml. were removed and 0.25 ml. of this whole suspension injected into each animal of Group 6. The remainder was centrifuged in an International refrigerated centrifuge at 4° C., 2,400 r.p.m. for 20 minutes. The supernate was carefully pipetted off and 0.25 ml. per mouse injected into Group 2. The residue was washed with 2 ml. of cold Locke's solution, gently stirred, recentrifuged, and the supernate discarded. Washing was repeated three times, after which 2.5 ml. of Locke's solution was added to half of the residue, which was thoroughly resuspended, and 0.25 ml. per mouse injected into Group 4. The remainder of the residue was recombined with and suspended in the balance of the original supernate, and 0.25 ml. injected into each mouse of Group 5. To prepare the suspension of lysed cells, marrow from 10 additional donors was removed into distilled water, after which 0.25 ml. was injected into each mouse of Group 3. All groups were sacrificed 25 days after the last irradiation; the tissues were dissected, weighed, and fixed for histologic examination as above.

Experiments 3a, 3b, and 3c were performed to determine whether bone-marrow injections are effective in promoting thymic recovery if injection is delayed until several days or more after irradiation. Suspensions were again prepared essentially as described, and injected intravenously on days 37, 45, or 50 (12, 20, and 25 days, respectively, after the last X-ray dose). Animals were sacrificed, together with their controls, on days 50, 59 or 67, and 63 or 69, respectively, and the dissected tissues weighed and studied as before.

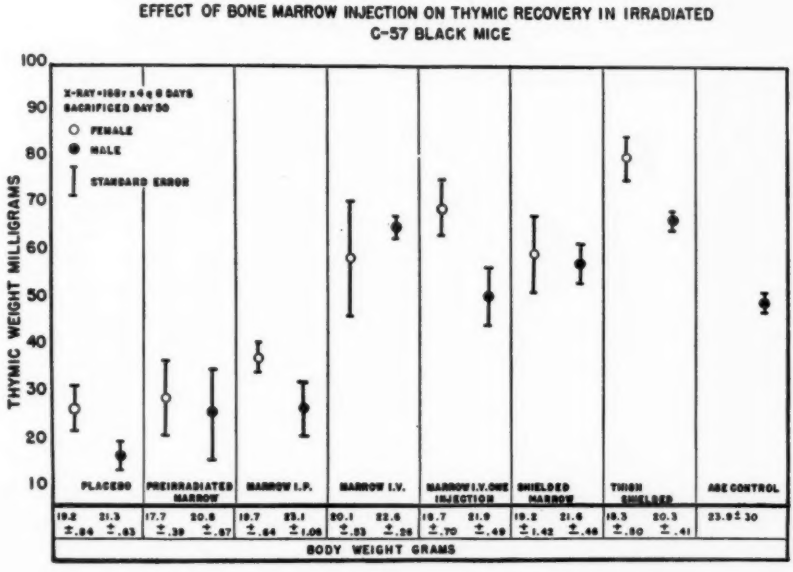
Experiment 4 was undertaken to determine whether spleen shielding promotes regeneration of the radiation-damaged thymus in the same way as thigh shielding or bone-marrow injection. Litter mates of both sexes were assigned to one of two groups and irradiated with 4 doses of 168 r at 8-day intervals. The spleen was exteriorized and enclosed within either a lead shield 4 mm. thick, or an identical container made of paraffin according to the method of Jacobson *et al.* (*4*). Originally, a series of 4 paired groups of 8 mice each was established, designated for sacrifice at experimental days 26, 30, 40, and 50. The irradiated animals tolerated repeated laparotomies poorly, however, and operative deaths sharply reduced the number of animals in some groups of both the shielded and sham-shielded series. As a result, all survivors in each series were pooled and sacrificed on day 51; the usual tissues were weighed and fixed for histologic study.

Experiment 5 was concerned with the effect of bone-marrow injections on lymphoid tumor incidence. In addition to 1 group of untreated age controls, 3 other littermate groups received the usual series of 4 irradiations, the thigh of one group being shielded with a lead strip 1 cm. wide and 3 mm. thick during treatment. The other 2 groups were injected intravenously after each irradiation, either with Locke's solution alone or with whole marrow suspended as before in Locke's solution, the injection volume being 0.25 ml. After experimental treatment was completed,

the animals were maintained under identical conditions and observed for the development of lymphoid tumors. All moribund and dead animals were autopsied, and representative tissues fixed for histologic study except where typical lymphomas were apparent on gross examination.

Results

Experiment 1.—The results are summarized in table 1 and mean thymic weights plotted in text-figure 2. Intravenously injected bone-marrow cell suspensions again accelerated thymic regeneration, though not to quite the same degree as thigh shielding. A single marrow injection after the last irradiation was just as effective as four postirradiation doses, but preshielded bone marrow had no greater effect than normal marrow. The effect of intraperitoneal injections of normal marrow is of borderline significance on the basis of thymic weight, but distinct histologic evidence of thymic recovery was noted as compared with the group receiving the Locke's solution placebo. The active principle of the marrow that is responsible for thymic regeneration was destroyed by preirradiation of the donor mice. Lymph nodes were also favorably affected by marrow injection, as indicated both by weight and microscopic picture. Although spleen weights were not significantly affected, histologic study rather consistently revealed more advanced regeneration of the white pulp in the thigh-shielded and normal-marrow-injected groups, and a greater degree of hematopoietic activity in the red pulp of placebo-injected animals. The lymphoid tissue responses were independent of body weight, as previously noted (*10*).



TEXT-FIGURE 2.

TABLE 1.—*Effect of bone-marrow injections on lymphoid tissue weights in irradiated C57BL mice**

Group	Postirradiation treatment	Thymus† (mg.)		Nodes† (mg.)		Spleen† (mg.)		Body weight† (gm.)	
		female	male	female	male	female	male	female	male
1	Locke's solution, I. V.	27.4±4.7‡	17.4±2.8	5.4±0.8‡	5.8±1.5	81.0±8.6‡	66.2±4.4	19.2±0.8‡	21.3±0.6
2	Preirradiated marrow, I. V., 4 injections	29.5±8.8	26.5±9.4	8.8±2.1	7.0±0.9	65.8±4.7	63.7±4.7	17.7±0.4	20.8±0.7
3	Marrow, I. P., 4 injections	38.2±3.0	27.4±5.3	12.7±0.9	14.1±1.9	87.2±7.0	86.7±12.9	19.7±0.6	23.1±1.1
4	Marrow, I. V., 4 injections	59.0±11.8	65.6±1.9	11.4±2.1	12.9±2.3	97.7±1.9	83.9±2.4	20.1±0.5	22.6±0.3
5	Marrow, I. V., 1 injection	69.5±6.5	51.2±6.2	14.7±1.3	13.7±3.0	90.5±4.2	79.7±2.9	18.7±0.7	21.9±0.5
6	Preshielded marrow, I. V., 4 injections	60.3±7.5	57.8±4.6	10.9±1.4	11.0±2.2	94.3±10.5	71.0±1.1	19.2±1.4	21.6±0.5
7	Thigh-shielded during irradiation	80.8±4.0	68.3±1.1	14.8±2.6	9.3±2.1	82.7±2.5	74.2±3.9	18.3±0.5	20.3±0.4
8	Age controls; no irradiation	.....	50.2±1.5	.....	15.4±1.2	.....	83.4±3.0	.....	23.9±0.3
9	Whole blood, I. V., 4 injections§	33.1±5.4	26.0±4.0	3.9±0.4	3.9±0.3	81.0±1.8	92.7±2.4	17.7±0.8	18.8±0.1

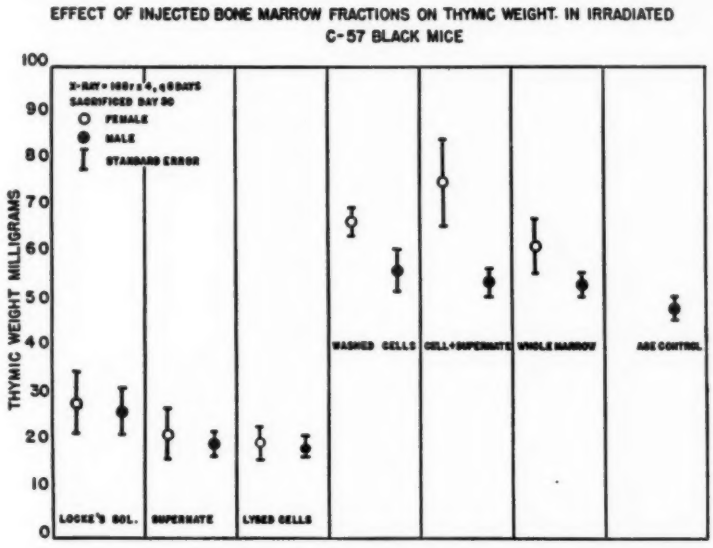
*All but Group 8 received 4 doses of 168 r each at 8-day intervals. All groups sacrificed on day 50.

†Mean and standard error by $\sqrt{\frac{\Sigma(\bar{x}-x)^2}{n(n-1)}}$, $n=4$ unless otherwise stated.

‡$n=3$.

§$n=2$.

Experiment 2.—Results are indicated in table 2 and mean thymic weights plotted in text-figure 3. Intravenous injections of washed cells alone, or of recombined cells and supernate, were just as effective as whole marrow in restoring thymic weight to normal levels. The supernatant fraction alone was without effect, and the activity of the marrow cells was destroyed by lysis in distilled water. These relationships were mirrored by the histologic appearance of the thymus in the various groups. Lymph node and spleen weights were not significantly affected by any treatment in this experiment.



TEXT-FIGURE 3.

Experiment 3.—Results are given in table 3. Cell suspensions of whole normal marrow injected intravenously 12 to 25 days after irradiation failed to influence thymic weights, as compared with placebo-injected and untreated controls. Centrifuged marrow residue and supernate were both ineffective when injected on day 45 (20 days after the last irradiation). Lymph node weights tended to be slightly greater in whole marrow and residue-injected groups, but the differences are not significant. Spleen weights exhibited no consistent trend.

Experiment 4.—Spleen shielding has the same effect on thymic weights as do intravenous bone-marrow injections (table 4); the significant increase over the sham-shielded thymic weights is obvious on inspection. Lymph nodes were also rather consistently heavier in the lead-shielded animals. Spleen weights were of course much greater, and histologic study revealed intense proliferative activity in the red pulp, as described by Jacobson *et al.* (*11*).

Experiment 5.—Results are presented in table 5, and cumulative incidence curves are shown in text-figure 4. All of the tumors were lympho-

TABLE 2.—*Effect of centrifuged bone-marrow fractions on lymphoid tissue weights in irradiated C57BL mice**

Treatment	Thymus† (mg.)		Nodes† (mg.)		Spleen† (mg.)		Body weight† (gm.)	
	female	male	female	male	female	male	female	male
1. Placebo	28.7± 5.1	26.7±4.3	6.4±1.5	5.8±0.7	68.1±5.4	70.9±3.2	18.1±0.8	23.4±0.8
2. Supernate	21.4± 4.6‡	19.7±1.5	7.9±1.7‡	7.8±1.3	70.4±1.8‡	56.1±2.5	19.4±0.2‡	21.6±0.3
3. Lysed cells	20.0± 3.2	19.0±2.5	8.2±2.6	4.2±0.9	69.5±6.0	59.7±3.9	18.6±0.7	20.4±0.4
4. Washed cells	67.3± 3.3	56.5±4.9	8.3±1.3	8.3±2.7	85.0±8.1	66.3±5.3	18.3±0.8	21.5±0.9
5. Recombined cells+supernate‡	76.5±10.7	53.8±3.7	9.7±2.4	8.4±1.4	73.8±4.4	73.1±3.3	17.8±0.5	20.8±2.1
6. Whole marrow	61.9± 5.1	53.5±1.5	9.3±1.2	5.7±0.5	76.7±1.8	75.7±4.4	18.5±0.5	22.8±0.8
7. Untreated age controls§	.....	48.8±1.9	.....	13.3±0.4	.....	80.7±3.7	.....	23.8±0.7

*Irradiation: 4 doses of 168 r each at 8-day intervals. All groups sacrificed on day 50.

†Mean and standard error by $\sqrt{\frac{\Sigma(\bar{x}-x)^2}{n(n-1)}}$, $n=4$ unless otherwise stated.

‡$n=3$.

§$n=7$; nonlittermate males.

TABLE 3.—*Lack of effect of delayed bone-marrow injections on lymphoid tissue weights in irradiated C57BL mice**

Group	No. of mice	Postirradiation treatment	Time of sacrifice	Thymus† (*mg.*)	Nodes† (*mg.*)	Spleen† (*mg.*)
I-a	8	Whole marrow, I. V., day 50.	Day 63...	42.3	9.6	63.7
II-a	6	None.....	Day 63...	38.0	7.2	62.6
III-a	13	Whole marrow, I. V., day 50.	Day 69...	40.0	10.3	62.3
IV-a	14	None.....	Day 69...	37.1	8.1	64.2
I-b	4	Washed cells, I. V., day 45.	Day 59...	31.8	11.9	56.3
II-b	4	Supernate, I. V., day 45..	Day 59...	20.9	13.3	77.7
III-b	7	None.....	Day 59...	23.7	9.0	59.1
IV-b	7	Washed cells, I. V., day 45.	Day 67...	33.8	11.6	65.5
V-b	7	Supernate, I. V., day 45...	Day 67...	33.9	8.0	57.4
VI-b	16	None.....	Day 67...	30.9	7.9	55.5
I-c	16	Whole marrow, I. V., day 37.	Day 50...	22.9	10.3	73.7
II-c	8	Locke's solution, I. V., day 37.	Day 50...	24.6	9.1	74.4

*All groups received 4 total-body X-ray doses of 168 r each on days 1, 9, 17, and 25.
†Mean values only. Group differences not significant by inspection.

TABLE 4.—*Effect of spleen shielding on lymphoid tissue weights in irradiated C57BL mice**

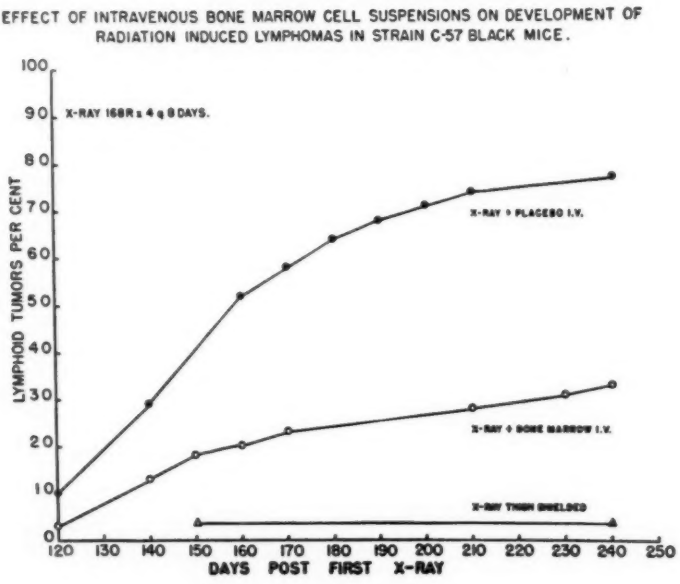
Group	Net no. of mice	Thymus † (*mg.*)	Nodes † (*mg.*)	Spleen † (*mg.*)
Spleen-shielded....	15	55.9 (34.5–82.0)	13.7 (6.8–19.5)	114.6 (85.5–159.6)
Sham-shielded.....	22	21.4 (7.4–42.7)	7.2 (3.1–13.3)	68.8 (12.5–98.5)

*Spleens exteriorized and enclosed in lead or paraffin, respectively, during each of 4 doses of 168 r at 8-day intervals.
†Mean and range of values.

TABLE 5.—*Comparative effect of intravenous bone-marrow injections and thigh shielding on development of radiation-induced lymphomas in C57BL mice*

Group	Treatment	Net no. of mice	Mice with lymphomas		Mean latent period	Dead negative	Alive
			(*no.*)	(%)	(*days*)		
I	X ray + Locke's solution, I. V..	31	27	*87*	165	1	3
II	X ray + bone marrow, I. V....	39	13	*33*	165	.....	26
III	X ray with thigh shielded.....	40	1	*3*	151	.....	39
IV	Untreated controls.....	20	0	0	.....	.....	20

cytic lymphosarcomas, identical with those previously described (*12*). As had been anticipated (*10*), intravenous injection of bone-marrow cell suspensions had a very distinct inhibitory influence on the development of lymphoid tumors, although the effect is significantly less than that of thigh shielding. This difference appears to be ascribable to the more rapid availability of the active principle from autologous marrow cells of the shielded femur than from injected, exogenous marrow.



TEXT-FIGURE 4.

Discussion

Based upon the results of these experiments and the previously cited work of other investigators, it is possible to characterize the active principle of bone marrow as follows:

a) It promotes regeneration of radiation-damaged hematopoietic tissues, although it does not prevent nor diminish the degree of initial radiation injury suffered by them.

b) It is radiosensitive and may be destroyed or inactivated by relatively low doses of radiation.

c) It is apparently not elaborated in greater amounts by shielded bone marrow; shielding therefore simply prevents the destruction or inactivation of the active material by radiation.

d) It is either not a readily water-soluble, extractable material, or is readily denatured upon removal from a living cellular environment. Cole *et al.* (*13*) found that the acute lethal effects of radiation could be significantly diminished by injection of the differentially centrifuged nuclear

fraction of cell suspensions of mouse spleen. The objection has been raised that these nuclear fractions are apparently contaminated with whole cells to the extent of perhaps 0.5 percent. Nonetheless, the absolute number of contaminating whole cells administered in their experiments would not appear to be sufficient to account for the effects observed. The activity may therefore be at a subcellular level but nonetheless closely associated with biologically active and readily denatured moieties.

e) The material is apparently not highly potent and is effective only when made available in relatively large quantities (*10*). Earlier work by Jacobson and co-workers indicates also that large amounts of splenic tissue must be implanted to provide protection against the lethal effects of irradiation (*11*). This would suggest that the active principle does not act as a catalytic agent but as a direct substituent for a deficiency existing in irradiated tissue. In this connection the work of Friedenwald and Sigelman (*14*) seems pertinent. These investigators found, in studying mitotic inhibition in the irradiated rat cornea, that the kinetics of inhibition were best explained on the basis of an induced deficiency of some material, an absolute amount of which appeared to be necessary to prepare cells for mitosis.

f) The material must be available to the irradiated animal very soon after treatment, though the exact time limits are not yet established. Injection of marrow as late as 12 days after irradiation is without effect. In related studies on postirradiation protection by parabiosis, Finerty *et al.* (*15*) concluded that the protective mechanism was effective between the third and tenth days after treatment.

g) The material is effective when given intraperitoneally, though distinctly less so than when given intravenously. In this respect its action on lymphoid tissue recovery parallels its action on radiation mortality. It appears that the material from shielded marrow or splenic red pulp reaches radiation-damaged tissues via the hematogenous route, but it has not yet been determined whether it must be transported intracellularly or can detach itself from its cells of origin and enter the blood as a truly humoral factor (*16*).

h) The material may not be species specific, in that Lorenz *et al.* have reported that guinea pig marrow may be protective for irradiated mice (*7*).

i) The material appears to be rather highly specific for bone marrow; it is apparently not present in significant quantities in thymus or muscle tissue (*10*), and was not demonstrable in whole blood in the small amounts used, although there is conflicting evidence that whole blood in large amounts may protect against the lethal effects of irradiation in dogs (*17, 18*). There is increasing evidence to support the view that the spleen of the mouse is an unusually active and sensitive organ with respect to hematopoiesis, and that its red pulp may be regarded as a large auxiliary reserve of extraskeletal marrow. Thus, the effects of thigh shielding and of spleen shielding on radiation-induced biological events might be expected to be at least qualitatively identical. From experiment 4, it is clear that spleen shielding has the same effect as marrow injection or

thigh shielding on thymic regeneration, and the recent work of Lorenz, Congdon, and Uphoff (*19*) demonstrates that spleen shielding can duplicate the striking inhibitory effect of thigh shielding on lymphoma induction.

j) The chemical nature of the active material cannot yet be clearly defined. Cole *et al.* (*13*) found that the activity of their nuclear fractions was destroyed by desoxyribonuclease, and are inclined to believe that the material is a desoxyribonucleoprotein complex, but much further work will be required to characterize the chemical nature of this substance definitively.

It seems clear that the effect of the marrow (and spleen) principle on thymic regeneration soon after irradiation is causally linked to its protective effect against lymphoid tumor development. This conclusion serves to explain the paradox, stated in the introduction, that local irradiation of the thymic region is ineffective in the induction of these tumors. During local irradiation, much bone marrow is of course preserved in the nonirradiated portions of the body, and by enabling early thymic recovery, this nonirradiated marrow effectively prevents lymphoma development. The injection of exogenous marrow can reproduce this effect but is somewhat less efficient. The difference between the effectiveness of endogenous and exogenous marrow is most likely ascribable to the time lag required for adaptation of the exogenous marrow to its new environment or for the utilization of its active principle. It remains to be established whether the effectiveness of exogenous marrow diminishes gradually or abruptly with increasing time after irradiation.

Perhaps the most important inference from these results is that lymphoid tumor development may be effectively blocked by a mechanism that does not in any way modify the degree of initial radiation injury suffered by the tissues in which the tumors would develop. Thus the initial radiation injury *per se* cannot be wholly responsible for tumor induction, and must be thought of as merely setting in motion a train of events that are still reversible by appropriate treatment during a finite time period after irradiation. At least two classes of agents are known to be effective in inhibiting tumor development during this postirradiation period: *a*) the active marrow (or spleen) principle, and *b*) certain hormonal influences (*20*, *21*). Further study of the complex interrelationships of these factors during the immediate postirradiation period seems strongly indicated.

Summary

In a series of experiments in irradiated C57BL mice, it has been shown that injection of cell suspensions of homologous bone marrow accelerates regeneration of the radiation-involuted thymus and lymph nodes and significantly inhibits the development of thymic lymphoid tumors. The active principle of the marrow is radiosensitive, is not readily water-extractable, and is associated with the centrifuged washed marrow cells. It is less effective when injected intraperitoneally than intravenously, and ineffective when given 12 days or more after irradiation. Spleen shielding has the same effect upon thymic regeneration, and has been shown by

Lorenz *et al.* (*19*) to block lymphoid tumor genesis. The failure of local irradiation of the thymus to elicit these tumors, and their inhibition by thigh shielding, are thus explained by the regenerative effect upon the thymus of intact nonirradiated marrow. Other implications of these results are discussed.

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Influences of Heredity and the Mammary Tumor Agent on the Occurrence of Mammary Tumors in Hybrid Mice[1]

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First-generation hybrid female mice derived from low-mammary-tumor strain females and high-mammary-tumor strain males usually exhibit a low incidence of mammary tumors because they do not acquire the mammary tumor agent in their mothers' milk. An exception to this rule was found when F_1 hybrids procured from strain BALB/c females and strain C3H males showed a high incidence (*1*). Following this observation a series of experiments was performed to elucidate the factors involved in the occurrence of these tumors.

The first approach consisted of efforts to detect an attenuated or latent agent in strain BALB/c animals (*2–5*). This appeared logical because, while the majority of tumors arose in old hybrids that were deficient in any demonstrable agent, a few appeared in very young hybrids that carried the agent. As the work progressed it became clear that the appearance of tumors in old mice was dependent upon hereditary influences and hormonal stimulation without the intervention of the agent, while the tumors in young animals arose after they had acquired the agent from their fathers.

This report records further studies on the influences of heredity and the mammary tumor agent on the production of tumors in F_1 hybrids born to strain BALB/c females. The importance of heredity was determined by mating BALB/c females to males of various strains, and the significance of the agent was studied by using males with or without the agent.

It was found that hybrids derived from strain BALB/c females and DBA/2 or RIII males showed a relatively low incidence of tumors and the presence of the agent in the male parent did not exert any appreciable influence upon the occurrence of tumors in old hybrids.

Materials and Methods

All mice were raised in this laboratory. They were maintained under similar environmental conditions, and given an unlimited supply of Purina laboratory chow and tap water. Females of strain BALB/c (formerly

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called strain C) when 2 to 4 months old were bred to males of strains C3H, DBA/2, or RIII of similar age. The females of the resultant F_1 hybrids were weaned when 1 month old and mated to agent-free males 1 to 2 months later. Each cast 3 litters in rapid succession and the litters were removed soon after birth. The mothers were then kept under observation and examined every other week for palpable tumors.

Histologic sections were prepared from every tumor. The tissues were fixed in Fekete's modification of Tellyesniczky's fluid (2 parts formalin, 20 parts 70 percent ethyl alcohol, and 1 part glacial acetic acid) and stained with hematoxylin and eosin.

Experimental Procedure and Results

Four experiments were performed, the results of which are summarized in table 1.

TABLE 1.—*Occurrence of mammary gland tumors in F_1 hybrid females derived from strain BALB/c females and strain C3H, DBA/2, or RIII males*

Experiment No.	Derivation of mice*	Group No.	Litter sequence	No. of mice	No. developed tumors	Tumor incidence (*percent*)	Average age at which tumors occurred (*months*)	Average age at death without tumor (*months*)
1	BALB/c × C3H+..	1	1 and 2..	73	36	49	24	24
1	BALB/c × C3H+..	2	3 and 4†..	64	43	67	22	24
1	BALB/c × C3H−..	3	1 and 2..	66	28	42	24	24
1	BALB/c × C3H−..	4	3 and 4‡..	35	18	51	21	23
2	BALB/c × C3H+..	1	1.....	20	12	60	22	26
2	BALB/c × C3H+..	2	2†.....	23	9	39	22	24
2	BALB/c × C3H−..	3	1.....	24	10	42	25	26
2	BALB/c × C3H−..	4	2‡.....	24	10	42	22	24
3	BALB/c × DBA/2+	1	1.....	15	1	7	23	23
3	BALB/c × DBA/2+	2	2†.....	24	9	38	16	23
3	BALB/c × DBA/2−	3	1.....	19	0	0	.....	22
3	BALB/c × DBA/2−	4	2‡.....	24	4	17	19	23
4	BALB/c × RIII+..	1	1.....	46	3	7	17	24

*A (+) sign after the male parent denotes agent-containing males; a (−) sign denotes agent-free males.
†Born to BALB/c females that had bred with agent-free males.
‡Born to BALB/c females that had bred with agent-containing males.

Experiment 1

This experiment was designed to answer two questions. First, do tumors appear in F_1 hybrids obtained from strain BALB/c females and strain C3H males when their mothers are known to be free of even an attenuated or latent agent? To obtain agent-free strain BALB/c females, a litter was removed from the mother by surgical procedure during the last 24 hours of gestation, and foster-nursed by a strain C57BL female. This technique is known to yield lines of mice devoid of the agent. The line of strain BALB/c thus procured has completed 6 generations of

brother-to-sister matings, and of 113 breeding females surviving to an average age of 19 months only 1 has developed a mammary tumor. This animal was 25 months of age.

Secondly, does the presence of the agent in the C3H male parent have a definite influence upon the incidence of tumors in the offspring? To find the answer, males from the C3H colony (*6*) and those from an agent-free line established in this laboratory (*7*) were used. The latter were from the F_{13} generation of the agent-free line.

If, in previous studies, offspring from strain BALB/c females developed tumors because they ingested the agent in mother's milk, then offspring born to BALB/c females used here should show a much lower incidence of tumors. If the agent in the male parent influenced the occurrence of tumors in the offspring, then those fathered by agent-carrying (C3H+) males should exhibit a higher incidence than those fathered by agent-free (C3H−) males.

Twenty-four strain BALB/c females were used to procure F_1 hybrids. After raising 2 litters, those mated to C3H+ males were bred to C3H− males and vice versa for an additional 2 litters. Thus, 4 groups of hybrids were obtained: the first belonged to first and second litters fathered by C3H+ males; the second belonged to third and fourth litters fathered by C3H+ males after the BALB/c females had bred with C3H− males; the third belonged to first and second litters fathered by C3H− males; the fourth belonged to third and fourth litters fathered by C3H− males after the BALB/c females had bred with C3H+ males.

It was hoped that this breeding program would reveal any influence due to litter sequence as well as revealing whether the father's agent can infect the offspring without infecting the mother. This latter aim could be attained if offspring born to a BALB/c female and C3H+ male contained the agent while those born in subsequent litters to the same mother and a C3H− male were deficient in the agent.

Of the 24 strain BALB/c females, 12 completed the breeding regime of 4 litters, 2 raised but 1 litter, and 10 raised 3 litters. This latter group consisted almost entirely of those that had bred first with C3H+ males but produced only 1 litter when bred to C3H− males. Failure to procure another litter from these matings accounts for the smaller number of hybrids in group 4 of this experiment.

The strain BALB/c mothers died at an average age of 20 months and none developed a mammary tumor. Results in the F_1 hybrids are shown in table 1. All groups confirmed previous findings with BALB/c × C3H hybrids. Those fathered by C3H+ males showed a higher incidence of tumors than those fathered by C3H− males, but the difference was not as pronounced as in earlier experiments (*5*) and was not considered significant.

Two littermate hybrids acquired the agent from their C3H+ father. They were members of group 2 that developed tumors when 5 and 6 months old. They were the only female members of the fourth and last litter born to their mother. When pieces of their spleens were implanted

subcutaneously into young mice susceptible to the agent, the test mice developed breast tumors within 12 months.

The strain BALB/c mother of the above mice was sacrificed when 17 months old and the agent was also detected in her splenic tissues. She had borne 6 hybrids by a C3H— male: 3 developed tumors at an average age of 21 months and 3 died free of tumor at a mean age of 24 months. Her third litter, fathered by a C3H+ male, contained 4 females: 1 had a tumor when 28 months old and 3 died without tumor at an average age of 23 months. These data indicate that the BALB/c female did not harbor the agent while nursing her first 3 litters, and although she acquired the agent later, she did not develop a breast tumor.

A hybrid of group 1 of this experiment that developed a tumor when 5 months old was, unfortunately, not tested for the agent. She belonged to a second litter fathered by a C3H+ male and had 2 sisters: 1 had a tumor when 18 months old and the other died without tumor at 23 months of age. Her mother was bred to a C3H— male and cast 2 litters, each of which contained 2 females; 3 of these developed tumors at a mean age of 21 months and 1 died without tumor when 8 months old.

The BALB/c mother was 22 months old when she was killed and her spleen tested for the agent. None of the test mice developed a tumor.

Analysis of the results according to litter sequence revealed that 65 hybrids of first litters had an incidence of 51 percent; 74 of second litters had an incidence of 43 percent; 65 of third litters an incidence of 56 percent; and 34 of fourth litters an incidence of 71 percent. These differences are not considered significant. More litters may be essential to demonstrate any influence of litter sequence.

A total of 126 mammary tumors was found in these mice. The tumors were sorted according to a scheme previously described (*4*, *8*) into: 1) adenocarcinoma, Type A, in which a reproduction of mammary acini is an outstanding feature; 2) adenocarcinoma, Type B, in which reproduction of mammary acini is not prominent but the morphology of the tumor is within the range of variation commonly observed in mammary tumors of mice; 3) adenocarcinoma, Type C, in which a characteristic morphology, consisting of small epithelial-lined cysts surrounded by fusiform cells that color yellow with van Gieson's stain, is found; 4) adenoacanthoma, in which a considerable amount of keratinization has developed; 5) carcinosarcoma, in which both carcinomatous and sarcomatous elements are found; 6) sarcoma of the mammary gland area, where sarcomatous tissue surrounds mammary ducts; and, finally, 7) a miscellaneous or unclassified group not fitting any of the above categories.

Using this classification, only 18 of the tumors were adenocarcinoma, Type A. This form of tumor is predominant in strain C3H mice with the agent, but may be relatively less frequent in other strains with the agent. Seventy-two were adenocarcinoma Type B, which is also frequent in mice with the agent. Twenty-two were adenocarcinoma Type C, a form of tumor which is very rare in mice with the agent but which we have observed rather frequently in old hybrid mice lacking the agent and having

one strain BALB/c parent. Three of these tumors were noted at autopsy to be buried within the muscle. One adenocarcinoma Type C was notable because it was combined with adenocarcinoma Type B. Adenocarcinoma Type C tumors nearly always present a uniform histologic pattern throughout the section. Nine tumors were adenoacanthoma, a form that has appeared very frequently in old C3H mice without the agent (*9*). Four of the adenoacanthomas resembled the molluscoid mammary tumors described by Haaland (*10*), and contained central cysts lined by keratinized epithelium. Six tumors were placed in the miscellaneous group. Two of these were composed of rows of epithelial cells in a cordlike arrangement, separated by considerable stroma. Three were composed of detached epithelial cells, a pattern that has been photographed previously [fig. 8A (*4*)]. The remaining tumor appeared to be an epithelial-lined cyst containing a single papillary ingrowth of tumor tissue. No carcinosarcomas or sarcomas of the mammary gland were found.

Analysis of the data revealed that the 5 types of mammary tumors were distributed evenly among the 4 groups of hybrids. Previous studies (*9*) have indicated that the morphology of mammary tumors varies somewhat with age. The average age of the mice bearing the different types of tumors was calculated, and the range for all groups was from 22 to 24 months, which cannot be considered a significant difference. When ages of the individual mice were considered, however, no adenocarcinomas Type C were found in mice below the age of 15 months, while 8 Type B tumors were in this age group. One adenoacanthoma, a remarkably good representative of the "molluscoid" form, appeared in a 5-month-old mouse of group 1. This tumor type has been found most frequently in old strain C3H mice without the agent.

In this experiment mammary tumors arose in F_1 hybrids derived from strain BALB/c females that were presumably free of the mammary tumor agent and C3H males with or without the agent. These results supplied answers to the 2 chief objectives of the investigation; namely, that an attenuated or latent agent is not involved in the high incidence of tumors in old hybrids, and the presence or absence of the agent in the male parent does not exert any pronounced influence on the incidence.

Experiment 2

The primary purpose of this experiment was to determine whether the presence or absence of the agent in strain C3H males of the same genetic constitution influences the occurrence of tumors in their BALB/c × C3H offspring. In all previous experiments of this kind the agent-free C3H males were members of a line separated from the C3H colony for a number of generations and, as mentioned in an earlier publication (*5*), their genetic constitution may have differed sufficiently from those of the colony males that their offspring were more resistant to the development of tumors.

In this study 4 C3H males, equally divided between 2 litters from the F_{15} generation of the agent-free line, were used. Within a few hours after

birth 1 male from each litter was transferred to a colony C3H female for the entire nursing period. The fostered males, which now contained the agent, were weaned when 1 month old and 1 month later they and their nonfostered litter mates, which were free of the agent, were used as breeders. In the breeding regime described below, littermate males were bred to the same strain BALB/c females.

After the hybrids were procured the seminal vesicles of these 4 males were tested for the presence of the agent by a technique described previously (*7*). The agent was detected in those fostered by the C3H females but not in those that remained with their agent-free mothers.

Thirteen strain BALB/c females were used: 7 were from the BALB/c line described in experiment 1, and 6 from the BALB/c colony (*2*). Since, as in experiment 1, hybrids born to these two groups of mothers showed similar incidences of tumors, they are all designated as BALB/c females in this experiment.

Each BALB/c female raised 2 litters; 7 bred with agent-free (C3H−) males, and 6 with agent-containing (C3H+) males for their first litters and with the litter mates of these males for the second litters. These matings produced 4 groups of hybrids: the first was of first litters born to BALB/c females and C3H+ males; the second from second litters born to C3H+ males after the BALB/c female had cast a litter by a C3H− male; the third from first litters born to BALB/c females and C3H− males; the fourth from second litters born to C3H− males after the BALB/c female had cast a litter by a C3H+ male. The reasons for this litter sequence were the same as those described for experiment 1.

All the mothers died without tumor at a mean age of 23 months. Results for the hybrids are shown in table 1. Those fathered by C3H+ males showed an incidence of 49 percent and those fathered by C3H− males an incidence of 42 percent. This difference is not significant. Analysis of the results according to individual fathers revealed that the offspring of each showed approximately the same incidence.

A total of 41 tumors was examined in this group, and the proportion of the different types of tumors was similar to that in experiment 1. Only 6 of the tumors were adenocarcinoma Type A, and 21 were adenocarcinoma Type B. Adenocarcinoma Type C tumors were relatively frequent, appearing six times. Three adenoacanthomas were listed. Five tumors were placed in the miscellaneous category. Two of these showed a papillary pattern, each papillary projection being covered by single layers of epithelial cells. Another was composed of multiple small cysts each lined with a single layer of epithelial cells. The fourth tumor was composed of remarkably large cells arranged in an irregular adenomatous pattern, and the fifth tumor showed some areas of an adenomatous pattern, which was largely obscured by a heavy infiltration of lymphocytes, and some areas of acanthoma.

The outcome of this experiment confirmed the findings of experiment 1. Tumors arose in hybrids procured from agent-free strain BALB/c females and C3H males with or without the agent. The use of littermate C3H

males here suggests that in earlier experiments the agent was not responsible for the higher incidences in hybrids fathered by C3H+ males.

Experiment 3

This experiment was similar to experiment 2 in all respects except the use of DBA/2 instead of C3H males. The DBA/2 fathers belonged to one litter of the F_{10} generation from a line of agent-free DBA/2 mice maintained in this laboratory (*7*, *8*). The primary purpose of the experiment was to ascertain whether hybrids procured from BALB/c females and DBA/2 males would also show a high incidence of tumors.

Thirteen BALB/c females were used and all died free of tumor at a mean age of 18 months. The seminal vesicles of the DBA/2 males were tested for the presence of the agent and, as in experiment 2, it was found only in those suckled by C3H females.

The outcome for the hybrids is shown in table 1. Of 9 tumors found in group 2 hybrids, 4 arose in mice from 6 to 10 months of age. These mice were the only females of a litter born to a BALB/c female and DBA/2+ male after the female had bred with a DBA/2− male. The agent was found in the spleen of the only hybrid tested for its presence. The BALB/c mother became ill and was killed when 15 months old, just 1 month before her last 3 offspring developed tumors. She was not tested for the agent. Her first litter was fathered by a DBA/2− male and contained 2 females, both of which died without tumor when 19 and 24 months old. These data indicate that the hybrids acquired the agent from their DBA/2+ father.

When this litter is deleted from group 2 of the experiment, the group shows a tumor incidence of 25 percent at an average age of 22 months. This, together with the incidences in other groups, permits the conclusion that, under the conditions of this experiment, the tumor rate in hybrids derived from BALB/c females and DBA/2 males is lower than that in hybrids from BALB/c females and C3H males.

A total of 14 tumors was examined in experiment 3. Four were adenocarcinoma Type A, and only two were adenocarcinoma Type B. Adenocarcinoma Type C tumors were especially frequent, numbering seven. There was one sarcoma.

Experiment 4

Females of strain BALB/c and males of strain RIII produced the hybrids for this experiment. The RIII males were descendants from a litter kindly supplied by Dr. W. H. Woglom in 1945. In 16 generations of brother-to-sister matings, 245 breeding females have shown a mammary tumor incidence of 95 percent at a mean age of 9 months.

The aim of the experiment was to ascertain whether the BALB/c × RIII hybrids showed a high incidence of tumors. Fifteen strain BALB/c females were bred to 4 RIII males. Each female cast but 1 litter.

The BALB/c females died free of tumors at a mean age of 23 months. The occurrence of tumors in the hybrids is shown in table 1. It is clear that these hybrids were more resistant to the development of tumors than either the BALB/c × C3H or the BALB/c × DBA/2 hybrids.

One had a tumor when only 9 months old. An unsuccessful attempt was made to find the agent in her spleen. She had 4 litter mates that died without tumor: 1 was 26 months old and 3 were 27 months old. Her tumor was diagnosed as an adenoacanthoma, which is rare in young mice carrying the agent. The other tumors found were an adenocarcinoma Type C in one mouse, and two Type C tumors in another.

Discussion

High incidences of tumors in the BALB/c × C3H hybrids of the first and second experiments confirmed previous experience with all such outcrosses made in this laboratory, and lower incidences in BALB/c × DBA/2 or BALB/c × RIII hybrids of the third and fourth experiments showed that, in the absence of the mammary tumor agent, the genic make-up of BALB/c × C3H hybrids was most conducive to tumor development.

This characteristic of BALB/c × C3H hybrids becomes more interesting when their incidence of tumors is compared with those of other F_1 hybrids lacking the tumor agent. In an earlier study (*7*) such F_1 hybrids derived from strains BALB/c, C3H, C57BL, I, and DBA/2 showed low incidences. Foulds (*11*) recorded incidences of 2 and 15 percent in C57BL × RIII hybrids; the lower incidence occurred in those obtained with agent-free RIII fathers. He also noted that tumors developed in C57BL × C3H hybrids but not in C57BL × A hybrids and concluded that "the strain of the father had a substantial effect on the incidence of tumours in F_1 hybrids." Mühlbock (*12*) studied C57BL × DBA hybrids and observed incidences between 0 and 14 percent. Three groups of 020 × DBA hybrids showed incidences of 8, 25, and 26 percent. Bittner (*13*) in a recent publication dealing primarily with transmission of the agent from the male to the female parent indicated that F_1 hybrids procured from agent-free strain BALB/c females and C3H+ males showed an incidence of 6 percent.[3] Hybrids derived from agent-free lines of strains A and C3H showed very low (0.1 and 1.1 percent) incidences. In a review (*14*) of experiments in which reciprocal breeding was carried out between high- and low-mammary-tumor strains of mice it was pointed out that F_1 hybrids derived from low-tumor strain females often showed higher incidences than the strain of the maternal parent. However, none of these incidences approached those of the BALB/c × C3H hybrids recorded here. It is concluded that the tendency to develop tumors is a unique characteristic of the BALB/c × C3H hybrids. Apparently their genetic constitution is such that, in the absence of the agent, mammary tumors are elicited by a relatively small degree of hormonal stimulation.

[3] The difference between Bittner's results with (BALB/c × C3H) F_1 hybrids and those reported here may be due to subline differences. After Bittner's BALB/c females became infected by the males, the incidence in the hybrids became 100 percent.

Findings in experiments designed to study the role of the mammary tumor agent in the development of tumors in the BALB/c × C3H hybrids showed clearly that their high incidence was not caused by the presence of an attenuated or masked form of the agent in the strain BALB/c females. This confirmed all previous efforts to detect the agent in strain BALB/c mice and gave unequivocal evidence that the agent is not responsible for the high incidence of tumors in old hybrids. It is, of course, possible that the BALB/c females harbor another type of agent which is not transmitted by way of mother's milk.

A discussion of the significance of the presence of the agent in the paternal parent of the hybrids must be divided between those hybrids that were free of the agent and those that acquired the father's agent.

As stated previously, males with or without the agent were used in the present experiments because a summary of earlier work suggested that agent-free F_1 hybrids fathered by agent-free C3H males had lower incidences of tumors than those fathered by males possessing the agent. In the present study both groups showed similar incidences of tumors. Mühlbock (*12*) performed the same type of experiment and obtained similar results. Foulds (*11*) found a higher incidence in hybrids fathered by males containing the agent, but this was the result of a high incidence in a few families that had acquired the father's agent. Apparently the the presence of the agent in the father does not alter significantly the incidence of tumors in agent-free F_1 hybrids.

Transmission of the agent from the male parent to F_1 hybrids was observed in a litter of BALB/c × C3H hybrids of experiment 1 and in a litter of BALB/c × DBA/2 hybrids of experiment 3. This confirmed previous findings with BALB/c × C3H hybrids and extended them by showing that BALB/c × DBA/2 hybrids may also acquire the agent from their fathers. In both instances the agent gained access to only a few hybrids and these were confined to 2 litters. Infection of a small number of hybrids also confirmed earlier work in this laboratory. Foulds (*11*) encountered similar results and attributed them to the "erratic transmission of tumour agent by the male parent." Mühlbock (*12*) concluded that "transmission of the agent by the male parent is possible, but seldom occurs." These conclusions are in harmony with the results reported here.

Bittner (*13*), however, found a high rate of transmission. When strain BALB/c females were bred to C3H males, 16 of 27 females developed tumors and a few that died without tumor transmitted the agent to their offspring. He included substrain differences and breeding procedures as possible explanations for his findings. Bittner drew attention to strain differences between females in their susceptibilities to infection by the agent from males, and strain differences between males in their abilities to infect females. F_1 hybrids that acquired the agent in this laboratory as well as those reported by Foulds and Mühlbock were fathered by males harboring the agent. The majority of Bittner's hybrids were also in this category but he cited observations that progeny born to agent-free parents also possessed the agent.

Thus far, Mühlbock *et al.* (*15*) have reported the only serious attempts to determine whether the agent from the male can gain access to the hybrids by intra-uterine infection of the embryo or whether the mother becomes infected first and then transmits the agent in her milk. Their results failed to show that the hybrids can acquire the agent *in utero*. As mentioned in an earlier discussion (*4*) evidence in this laboratory suggests that, on occasion, offspring may be infected *in utero*. This problem is of considerable interest and is receiving attention.

Summary

A high incidence of mammary tumors in F_1 hybrids derived from low-mammary-tumor strain BALB/c females and high-mammary-tumor strain C3H males led to a series of experiments to ascertain the roles of heredity and the mammary tumor agent in the production of these tumors. In order to minimize the influence of hormonal stimulation each hybrid was force-bred and permitted to cast 3 litters.

When hybrids were produced by mating strain BALB/c females to males of strain C3H, DBA/2, or RIII, those born to C3H fathers showed high incidences of tumors whereas those born to DBA/2 or RIII fathers showed lower incidences. Apparently a high degree of susceptibility to the development of mammary tumors in the absence of the agent is a unique characteristic of the BALB/c × C3H hybrids.

Mammary tumors arose in BALB/c × C3H hybrids derived from strain BALB/c females that were deprived of the possibility of carrying the agent in an attenuated or masked form.

Hybrids obtained from BALB/c females and C3H males with or without the agent showed similar incidences of tumors. The presence of the agent in the male parent does not exert a significant influence upon the occurrence of these tumors when the hybrids do not acquire the agent from their fathers.

The mammary tumors arising in these mice were examined microscopically and sorted into types. No significant differences were found in the types of tumors occurring in the different experimental groups. Adenocarcinoma, Type C, was remarkably frequent in the mice in all experiments.

The agent appeared in one litter fathered by a strain C3H male and in one litter fathered by a DBA/2 male. Both males carried the agent. Thus, a few hybrids born to strain BALB/c females and C3H or DBA/2 males may acquire the agent from their fathers, but the large majority of BALB/c × C3H hybrids develop tumors in the absence of the agent.

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Responses of Strain DBA$_t$/2 Mice, Without the Mammary Tumor Agent, to Oral Administration of Methylcholanthrene[1]

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In a discussion of results (*1*) of experiments in which mammary gland tumors and leukemia were elicited in strain DBA$_t$/2 female mice by percutaneous applications of methylcholanthrene, it was stated that the licking habits of mice raised the question whether the tumors could be induced by ingested carcinogen. A survey of the literature revealed that while various investigators (*2–14*) had used the oral route for exposing mice to carcinogens, none had reported the production of mammary tumors or leukemia.

Van Prohaska, Brunschwig and Wilson (*15*) observed 4 breast tumors in 15 mice that had been force-fed a solution of methylcholanthrene in olive oil. They reported the occurrence of such tumors in untreated females of the stock and pointed out that all the tumors in their experimental animals arose forward of the thorax, an area over which the oil solution spread following oral administration. Orr (*16*) found that intranasal application of methylcholanthrene produced mammary tumors in an inbred strain of mice. Shay and colleagues (*17–19*) reported that gastric instillation of large doses of methylcholanthrene over considerable periods of time induced adenocarcinoma of the breast and leukemia in Wistar rats.

This report deals with the results of oral administration of methylcholanthrene to DBA$_t$/2 mice that were free of the mammary tumor agent. It was found that leukemia, chiefly of a lymphocytic type apparently originating in the thymus, and referred to as mediastinal lymphosarcoma, was induced in both sexes. In addition, mammary tumors were induced in females.

Materials and Methods

Agent-free strain DBA$_t$/2 mice were used. They were members of a line described previously (*1*) in which breeding females show an incidence of less than 1 percent of mammary tumors and an incidence of less than 10 percent of spontaneous leukemia. The mice were 2 to 4 months of age

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when they received the first application of carcinogen. All were maintained as virgins in the same environment and fed unlimited quantities of Purina laboratory chow and tap water.

One week prior to the first application of carcinogen approximately half of the females and all the males received a 2-mg. stilbestrol-cholesterol pellet in the subcutaneous tissues of the right axilla. Females received pellets containing 0.5 or 1.0 percent stilbestrol and males 5.0 or 10.0 percent stilbestrol.

A technique of forced feeding described by Morris and Thompson (*20*) was used. Each mouse received 1 mg. of methylcholanthrene (m.p. 179–180° C.) dissolved in 0.2 cc. of olive oil at weekly intervals for 9 weeks. Thus, each received a total of 9 mg. of the hydrocarbon. They were then kept under observation for the occurrence of tumors. The experiment was terminated 1 year after the first feeding, at which time 11 males survived.

Histologic sections were prepared from virtually all tumors reported here. The tissues were fixed in Fekete's modification of Tellyesniczky's fluid (2 parts formalin, 20 parts 70 percent ethyl alcohol, and 1 part glacial acetic acid), blocked in paraffin, cut, and stained with hematoxylin and eosin.

Results

The findings are summarized in table 1. Males receiving 5.0 or 10.0 percent stilbestrol pellets gave similar results and are combined. It is clear that mammary gland tumors, leukemia, and pulmonary tumors were induced by the carcinogen.

Mammary gland tumors occurred in females only and, as in the earlier study (*1*), arose in those exposed only to methylcholanthrene as well as in litter mates exposed to both stilbestrol and methylcholanthrene.

Microscopic sections were prepared from 29 subcutaneous tumors. Two of these (described below) were papillomatous, and were derived from the skin appendages.

Twenty-seven tumors appeared to be derived from some element of the mammary gland, and on the basis of their histologic features they have been separated into groups according to a scheme previously described (*1*). Five were diagnosed as adenocarcinoma Type B, 1 as adenoacanthoma, 7 as mammary sarcoma, and 13 as carcinosarcoma. Several of the carcinosarcomas were composed principally of adenocarcinomatous tissue, but small areas were found where the carcinoma cells or the stroma appeared to be developing into fusiform sarcoma cells. The remaining tumor was unusual (figs. 1 and 2). The central area of the tumor was made up of branching strands of basophilic cells unlike any type found in the usual mammary tumors. Nests of rounded, more eosinophilic cells, however, such as frequently appear in mammary tumors, were found at the periphery. The pattern of this tumor somewhat resembled that of a tumor previously described and illustrated [(*1*) fig. 3B], which also developed in a $DBA_t/2$ mouse treated with methylcholanthrene.

The two papillomatous tumors appeared to be derived from a hair follicle and a sebaceous gland, respectively (figs. 3 and 4). Hair-follicle re-

TABLE 1.—*Occurrence of mammary gland tumors, mediastinal lymphosarcomas, and pulmonary tumors in strain DBA$_1$/2 mice following oral administration of methylcholanthrene*

Sex	Concentration of stilbestrol in a 2-mg. stilbestrol-cholesterol pellet (*percent*)	Number of mice	Response												
			Mammary gland tumors			Types and number of mammary gland tumors					Mediastinal lymphosarcomas			Pulmonary tumors	
			Number of mice	Tumor incidence (*percent*)	Average age (*months*)	Adenocarcinoma	Adenoacanthoma	Carcinosarcoma	Sarcoma	Miscellaneous	Number of mice	Tumor incidence (*percent*)	Average age (*months*)	Number of mice	Average age (*months*)
F	No pellet	44	14*	32	9	3	1	7	4	.....	26	59	8	2	14
F	0.5	9	5	55	9	.....	.....	2	3	.....	4	44	9	.....	.....
F	1.0	17	7	41	8	2	.....	4	.....	1	7	41	7	.....	.....
M	5.0 or 10.0	38	.....	.....	.....	.....	.....	.....	.....	.....	15	39	9	8	14

*One mouse had 2 tumors: an adenoacanthoma and a carcinosarcoma.

production was suggested by small cysts lined with flattened squamous cells, and sebaceous-gland reproduction by the presence of large, clear, foamy, pale cells which were found either singly or in groups.

Lymphocytic leukemia, as it occurred in the mice of this experiment, was particularly interesting because it was localized to the mediastinum and occurred in both sexes. Microscopic sections were available from 54 of the mediastinal masses. Fifty-two of these were similar and were diagnosed as lymphocytic neoplasms apparently originating in the thymus. All showed the same type of neoplastic cell which had the characteristic features of a malignant lymphocyte. The normal structures of the thymus were replaced by accumulations of these cells, but remnants of Hassall's bodies, and the original margin of the thymus could often be recognized. The neoplastic cells had extended beyond the borders of the thymus in all cases, often surrounding the aorta and extending into the hilus of the lung and the base of the heart. The outline of the tracheobronchial lymph nodes could sometimes be recognized, although the normal tissue was entirely replaced by neoplastic cells. Only a few collections of normal lymphocytes remained. The neoplastic process was always too advanced to determine the exact site of origin, but it was considered to be probably the thymus.

Pieces from 4 of these mediastinal masses were transplanted subcutaneously into a total of 15 F_1 hybrids derived from strains BALB/c and $DBA_t/2$. The transplants grew progressively in all inoculated animals.

Of the two remaining mediastinal masses (not diagnosed as lymphocytic neoplasms), one was found in a 9-month-old male and showed a general intermingling of large lymphocytic cells and reticulum cells. This form of neoplasm is not infrequent in the mouse and presents a problem in differential diagnosis between a lymphocytic neoplasm and a reticulum-cell sarcoma. The diagnosis finally accepted was reticulum-cell sarcoma. The second mass occurred in a 4-month-old male and contained a small group of neoplastic cells which were generally fusiform, except where the cells had invaded vessel lumina, in which location the cells were rounded and detached. This tumor was also diagnosed as reticulum-cell sarcoma. This type of tumor is very rare in mice of this age.

Four females (2 given estrogen and 2 not given estrogen) developed both mammary gland tumors and mediastinal lymphosarcomas.

Generalized infiltration of many organs with neoplastic lymphocytic cells developed in only 5 mice. One was a 15-month-old female not given estrogen, 1 a 15-month-old female that had been given estrogen, and 3 were males, 9, 13, and 15 months of age. The occurrence of lymphocytic neoplasms including those of mediastinal origin was observed in agent-free $DBA_t/2$ female mice of the earlier skin-painting experiments (*1*).

Pulmonary tumors were next in frequency. These were found only in mice over 11 months of age and showed the characteristic morphology (*21*). One pulmonary lesion, found in a 15-month-old male, was diagnosed as pulmonary adenomatosis. It was similar to the lesion of this type recently described by Horn *et al.* (*22*).

One of each of the following tumors was diagnosed in this group of mice: granulosa-cell tumor of the ovary, squamous-cell papilloma of the skin, hemangioendothelioma of the mesentery completely encircling the mesenteric lymph node, and reticulum-cell sarcoma in the wall of the uterus.

Discussion

These results confirmed the susceptibility of agent-free strain $DBA_t/2$ mice to the induction of mammary tumors by methylcholanthrene and showed that this type of tumor was produced by oral applications of the carcinogen. The morphology of the mammary tumors was notable because no adenocarcinoma with a predominant acinar structure was found, and that is generally the most frequent tumor in various strains of mice with the mammary tumor agent. The high incidence of carcinosarcomas and sarcomas of the mammary gland area was striking, and this modification in the morphology of the tumors in strain $DBA_t/2$ may be directly related to the methylcholanthrene exposure.

Oral administration is preferable to skin painting for inducing breast tumors in this strain of mice when the occurrence of skin tumors is undesirable. In previous experiments (*1*) a 0.25 percent solution of methylcholanthrene in benzene was applied to seven different sites to avoid the production of skin tumors but, despite the precaution, 54 of 158 mice developed skin tumors.

The high incidence of localized mediastinal tumors in the $DBA_t/2$ mice was of considerable interest. This form of lymphoma was found in $DBA_t/2$ females following topical applications of the carcinogen (*1*) and attention was directed to its prevalence in mice of this laboratory when the incidence was compared with the results reported by other investigators. It was suggested that substrain susceptibility, age of the test animals, or the dosage of carcinogen may have been responsible for the divergent findings. The present work shows clearly that the oral route of administration of methylcholanthrene is efficacious for the production of this type of lymphoma in $DBA_t/2$ mice.

The susceptibility of mice to induced mediastinal lymphosarcomas has been observed by various workers. The reader is referred to a review by Kirschbaum (*23*) and to recent papers by Kirschbaum, Shapiro and Mixer (*24*) as well as by Rask-Nielsen (*25–27*) for further information. Apparently, the induction of leukemia in mice following oral administration of a carcinogen has not been reported previously.

Summary

Agent-free strain $DBA_t/2$ mice of both sexes were given 9 weekly injections by stomach tube of 1 mg. of methylcholanthrene dissolved in 0.2 cc. of olive oil.

Mammary gland tumors were induced in the females, and leukemias—chiefly mediastinal lymphosarcomas—were induced in both sexes.

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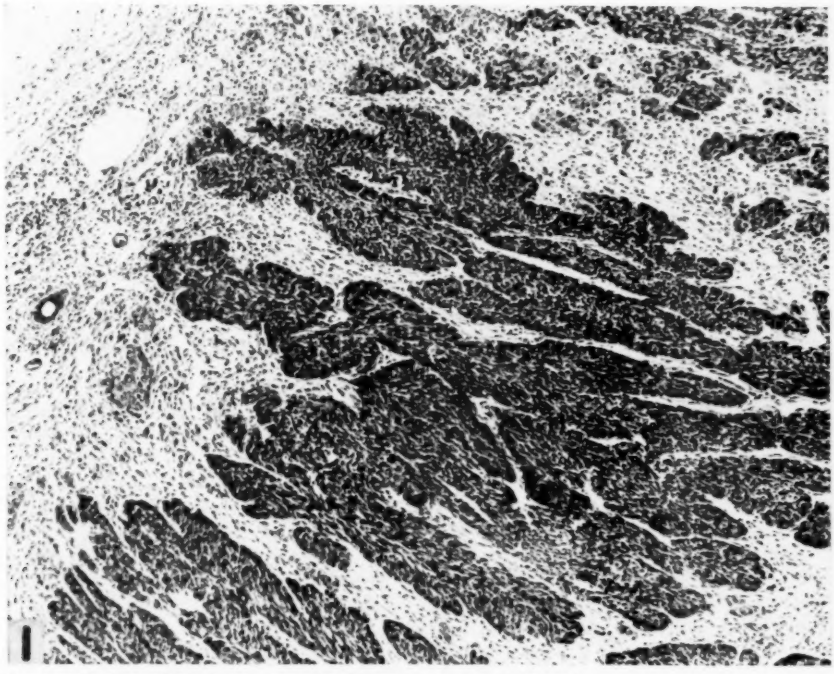
PLATE 25

FIGURE 1.—Tumor of unusual structure originating in area of mammary gland. Tumor mostly composed of sheets of basophilic cells, which are rounded at the margin of the sheets and fusiform toward the center. The duct of a mammary gland, and a nest of rounded, more eosinophilic cells are seen on the left at the periphery of the tumor. Hematoxylin and eosin. × 100

FIGURE 2.—Higher-power view of figure 1. Groups of rounded, more eosinophilic cells, which are often seen in the usual mammary tumor, appear in the center of the field. Sheets of basophilic cells are at bottom. Hematoxylin and eosin. × 260



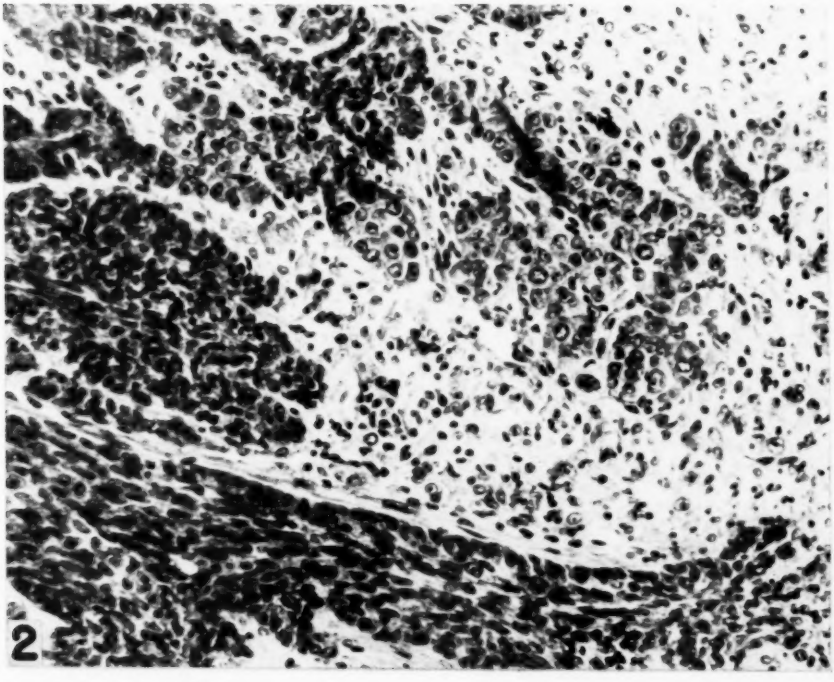


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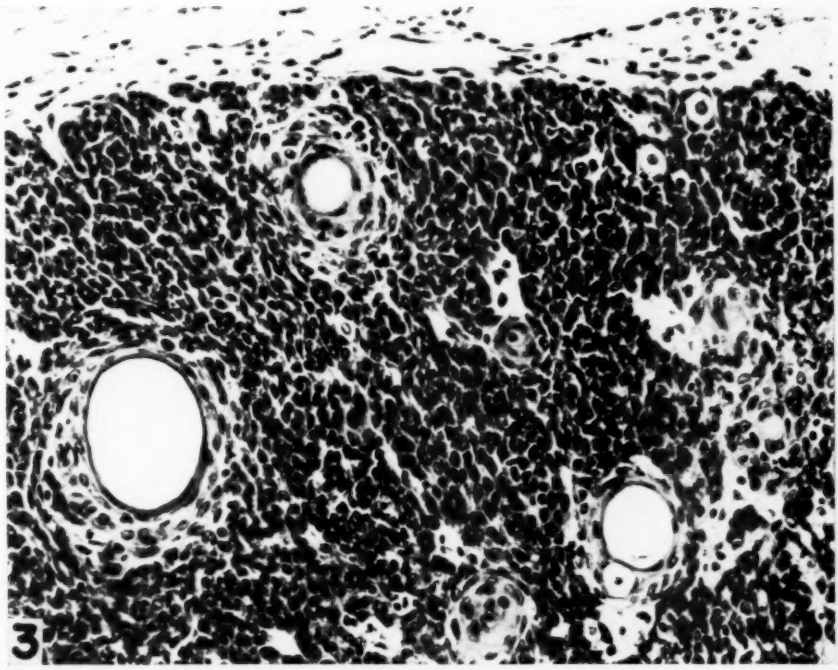
PLATE 26

FIGURE 3.—Tumor showing occasional clear sebaceous cells, and spaces lined by flattened epithelial cells reproducing hair shafts. Hematoxylin and eosin. × 260

FIGURE 4.—Tumor showing numerous nests of sebaceous cells, and small spaces lined by flattened epithelial cells. Hematoxylin and eosin. × 260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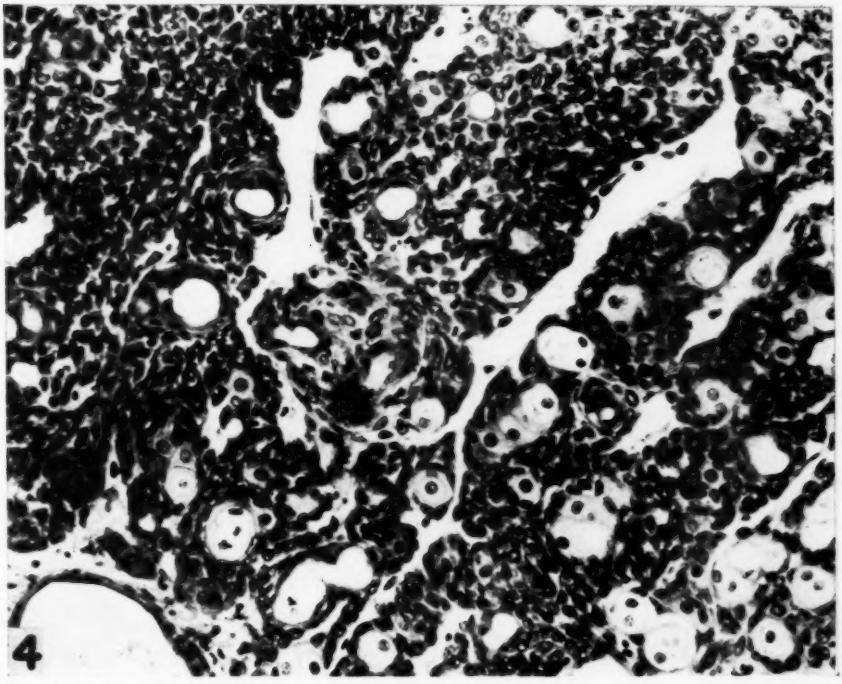
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Sulfhydryl Groups in the Skin of the Mouse and Guinea Pig [1, 2]

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The new technique of Barrnett and Seligman (*1*) demonstrates with greater clarity than before the distribution of alcohol- and water-insoluble, protein-bound sulfhydryl groups in tissues (*2*). This paper reports the distribution of these sulfhydryl groups in the skin of the mouse and guinea pig during the cycles of hair growth. Previous investigators have studied sulfhydryl groups in tissues principally with the use of potassium ferricyanide or nitroprusside, techniques having dubious specificity and weak color reaction. Sulfhydryl groups have been studied in skin by Giroud and Bulliard (*3–5*), by Giroud and Leblond (*6*) and by Chèvremont and Frederic (*7*) and Frederic (*8*). Hardy (*9*), using potassium ferricyanide and nitroprusside, studied sulfhydryl groups in the hair follicle of the mouse during different stages of activity. None of these authors, however, has correlated the distribution of sulfhydryl groups in the whole skin with the different stages of hair-follicle activity.

The morphology and physiology of the skin vary during the cycles of hair growth. Chemical irritants, for instance, induce different responses on the skin when hair follicles are growing than when they are resting (*10, 11*). When skin is X irradiated under these two conditions it responds differentially (*12*). Glycogen (*13, 14*) and acid polysaccharides (*15*) show fluctuations in amount that parallel the activity of hair follicles. It will be shown in this paper that the localization and concentration of sulfhydryl groups in skin also undergo changes during periods of activity and rest of the hair follicles.

Materials and Methods

Ten mice of strain BUC White and ten of strain C57BL were used in this study. All animals were between the ages of 4 and 6 months. In order to initiate growth of the next hair generation, an area on the dorsum of each mouse was plucked of its club hairs (*16*). Biopsy specimens were excised from each animal at 3, 4, 6, 9, 12, 18, 20, 21, and 26 days after

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plucking. Sulfhydryl groups were studied also in the skin of normal guinea pigs and in the sole pads, the esophagus and tongue of normal mice and in one epithelioma from a hairless mouse.

Small pieces of tissues were fixed for 24 hours in a solution containing 1 gm. of trichloroacetic acid dissolved in 100 ml. of 80 percent alcohol. The tissues were embedded in paraffin, sectioned at 10 μ and stained with Barrnett and Seligman's method (*1*) for the demonstration of protein-bound sulfhydryl groups, and mounted in glycerin. Sections are treated with 2,2′-dihydroxy-6,6′-dinaphthyl disulfide, which reacts with protein-bound –SH groups in tissues to form a colorless oxidation product. This substance is converted into a colored azo dye by coupling with tetrazotized diorthoanisidine. The most important caution in using this method is that the coupling agent is extremely unstable in solution, especially in sunlight, and must be used *immediately* after preparation.

When tissues are treated with the method of Barrnett and Seligman (*1*), a pink or red color indicates sparse, widely separated sulfhydryl groups, whereas a blue color indicates more concentrated sulfhydryl groups.

The results obtained with this method were compared with sections of tissues fixed in 10 percent neutral formalin and stained with the red sulfhydryl reagent, 1-(4-chloromercuriphenylazo)-naphthol-2 of Bennett (*17*). The results obtained with the two methods are comparable but the method of Barrnett and Seligman (*1*) gives a sharper color differentiation.

Observations

Three basic changes occur in the hair follicle during its developmental cycle: *anagen*, the period of active cell proliferation and development; *catagen*, the brief transition phase when cell proliferation ceases, hair follicles become shorter, and the hair club is formed; and *telogen*, the resting stage (*18*). The observations presented in this paper correlate the distribution of sulfhydryl groups in the skin of the mouse with the six critical substages of anagen of Chase *et al.* (*16*), and with catagen and telogen.

The skin of normal mice.—In skin with resting hairs, the short hair follicles and their dermal papillae lie within the corium. The Malpighian layer is two cell layers thick and the stratum granulosum is discontinuous. After staining for sulfhydryl groups, the Malpighian layer is colored a pinkish blue and the stratum corneum a light pink. The keratohyalin granules in the stratum granulosum remain unstained. In the pilosebaceous elements, the external sheath, like the epidermis, is colored a pinkish blue. The cortex of the hair is unreactive throughout its length. In and around the brush of the hair club, the keratinized cells are colored blue (fig. 1). Sebaceous glands stain light pink. The cells of the rounded dermal papilla are unstained. The panniculus carnosus stains light pink.

One day after telogen hairs have been plucked—anagen I of Chase *et al.* (*16*)—the cells of the "germ plate," which lie just above the rounded dermal papilla, divide mitotically and the plate becomes larger. After

2 to 3 days (anagen II), the germ plate grows downward around the upper end of the papilla, forming an inverted cup of epithelial cells—the hair bulb. After 4 days (anagen III), the bulb completely encloses the enlarging papilla and is producing an internal root sheath. At this time, the cells of the external sheath proliferate and the follicle grows downward. Coincidental with these changes, the epidermis attains a thickness of three times that of telogen (*19*).

In skin with hair follicles in anagen I through III, the Malpighian layer of the thickened epidermis stains blue with the sulfhydryl reagent and the stratum corneum stains light blue (fig. 2). The lower part of the stratum corneum immediately above the stratum granulosum often shows a stronger blue reaction. The ground cytoplasm of the cells of the stratum granulosum is of a pale blue color but the keratohyalin granules remain unstained. In the sebaceous glands, the indifferent peripheral cells stain blue while the more mature, centrally located cells contain reddish granules between the lipid droplets. The external sheath of the elongating hair follicle stains a light blue, while the internal sheath stains pink. The cells of the inverted cup of the hair germ stain blue but the dermal papilla remains unstained. In anagen III, the hair bulb and the dermal papilla are colored pink. The panniculus carnosus, however, stains a bright blue.

Hair proliferation begins at anagen IV or approximately 6 days after plucking. The hair shaft has both a cortex and medulla and the cuticles of the internal sheath and the hair cortex are clearly seen. The tip of the hair, which is composed of cortex and cuticle, extends to about the level of the sebaceous gland. The epidermis recedes to a thickness of 1 or 2 layers of cells and remains thin for the duration of anagen (*16, 19*). After treatment with the sulfhydryl reagent the Malpighian layer stains pink, as it does also for the remaining period of anagen. In the middle third of the follicle (fig. 3), the cortex begins to show an intense blue color in the parakeratotic zone of the hair root or "keratogenous zone" of Giroud and Bulliard (*3*). The reaction stops abruptly just above this area and the keratinized cortex remains unstained. The medulla stains a light pink. The panniculus carnosus stains pink.

By anagen VI, all of the formative stages of the follicle have occurred; this is the phase of rapid hair growth which continues for the next 8 or 9 days. The external sheath of the follicle stains pink. Huxley's layer and the cuticle of the internal sheath, around the "keratogenous zone," stain red and remain reactive up to the level of the base of the sebaceous gland. The trichohyalin granules in Huxley's layer are unreactive. Henle's layer, which stained red in C57BL mice, was unreactive in BUC White mice. The cells in the bulb stain red. This color changes progressively to a deep blue in the "keratogenous zone" of the cortex (figs. 4 and 5). The deep blue color disappears abruptly in the keratinized cortex of the hair above the "keratogenous zone." The cells of the cortex from the bulb to the "keratogenous zone" contain parallel fibrils that also stain blue. The medulla stains pink in the bulb, red in the "keratogenous zone" and pink in the rest of the hair. The cells around the papilla cavity, and the

cells of the elongated dermal papilla stain red. Hair follicles of C57BL mice show a stronger color reaction than do follicles of the BUC White strain.

Eighteen to 19 days after plucking, the follicles go into catagen. Hair proliferation ceases abruptly and the epidermis attains a thickness of 2 to 3 layers of cells. Hair follicles shorten to about one-third their previous length, cell proliferation in the bulb ceases, and a club is formed at the end of the hair. During this period the external and internal sheaths of hair follicles give a stronger sulfhydryl reaction than during anagen. The brush of keratinizing cells forming the hair club stains a bright red, while the incompletely keratinized cortex immediately above it is deep blue. The reaction ends abruptly above the club and the rest of the cortex remains unstained (fig. 6). The medulla of the entire hair stains a light pink. The epithelial cells below the club and the dermal papilla remain unstained. Catagen is completed by approximately 21 days, when hair follicles reach the telogen or resting stage.

The skin of the guinea pig.—The hair follicles of the guinea pig are larger than those in the mouse and the localization of sulfhydryl groups is even more striking than in the mouse. During late anagen and telogen the distribution of sulfhydryl groups in the hair follicles is similar to that found in hair follicles of corresponding stages in the mouse. In late anagen the "keratogenous zone" is a brilliant blue (figs. 7, 8, 9). As in the mouse, the reaction ends suddenly just above this zone (fig. 9). The medulla of the hair stains red. Henle's and Huxley's layers of the internal sheath stain pink, the cuticle stains a bright red color which begins at the "keratogenous zone" and extends to the base of the sebaceous gland. The external sheath is light blue, the cells in the hair bulb and the dermal papilla stain red. The epidermis is uniformly reactive, staining a reddish blue, but the keratohyalin granules in the stratum granulosum are unstained. In some specimens the lower layer of the stratum corneum just above the stratum granulosum stains red. The cells in the sebaceous glands contain many reddish-blue granules. In telogen or resting follicles, the external sheath is a reddish-blue color. The cortex of the hair is unstained but the brushlike projections of the club are surrounded by a deep blue band (fig. 10). All of the medulla stains pink.

The epithelioma.—The single epithelioma used in this investigation is composed of thick, digitiform cords of epithelial cells which are covered by a deep parakeratotic layer. There is no stratum granulosum separating the epidermis from the parakeratotic layer. After staining with the sulfhydryl reagent, the epidermis is colored blue and the parakeratotic layer an even more intense blue. The most intense reaction is obtained in the parakeratotic layer on the surface of the tips of the long epidermal projections (fig. 11). The nuclei of the parakeratotic cells also stain blue.

Miscellaneous.—In the tongue and esophagus the epithelium stains blue but the parakeratotic layer that covers it stains a darker blue. In the sole pad (fig. 12), the Malpighian layer stains blue, the stratum corneum stains a deeper blue, but the keratohyalin granules in the stratum

granulosum remain unstained (fig. 13). The small sweat glands in the sole pad show a particularly strong reaction in the luminal borders of the secretory cells (fig. 12).

Discussion

The shortcomings of the nitroprusside and the ferricyanide methods have been emphasized by Barrnett (*2*) and need not be restated here. The more specific method of Barrnett and Seligman (*1*), with its sharper color reactions, makes possible a more precise localization of thiol groups in the skin.

In the skin of the mouse during the early stages of anagen the hair follicles become elongated and form a hair bulb, and the surface epidermis becomes thicker (*19*); all of these structures show an abundance of sulfhydryl reaction. In anagen IV, as hair follicles begin to produce hair, the epidermis becomes thinner and its concentration of sulfhydryl groups decreases. During anagen VI, the greatest concentration of sulfhydryl groups in the skin is localized in the cortex and its cuticle in the "keratogenous zone" of the hair follicles; neither of these structures is reactive above this region. Although Giroud and Bulliard (*5*), Chèvremont and Frederic (*7*) and Hardy (*9*) failed to demonstrate the presence of sulfhydryl groups in the internal sheath of the hair follicle or in the medulla of the hair, we have shown in the skin of the mouse and guinea pig that the cuticle of the internal sheath and Huxley's layer are moderately reactive, but Henle's layer is barely reactive. In the guinea pig, the color reactions are stronger than in the mouse. The medulla of the hair stains pink throughout most of its length. These observations on the growing hair follicle are in general agreement with those of Barrnett (*2*).

During catagen, the most intense –SH reaction is in the developing hair club, and the external sheath shows a stronger –SH reaction than during anagen. In resting (telogen) hair follicles the high concentration of –SH groups is only around the brush of the hair club.

In growing hair follicles, then, the strongest reaction for sulfhydryl groups is obtained in the "keratogenous zone." However, free –SH groups, not demonstrated by this technique, might also be present in the hair bulb and in the dermal papilla. Since glutathione plays an important role in cell division, it could be present in the matrix cells of hair follicles (*20, 21*). There might also be a rapid turnover of an –SH amino acid or polypeptide which becomes incorporated into the fibrous protein.

The stratum corneum in the epidermis of the mouse is strongly reactive during early anagen, but during the remainder of the hair growth cycle it is only moderately so. In the skin of the guinea pig the stratum corneum was always reactive. Giroud and Bulliard (*5*) and Chèvremont and Frederic (*7*) failed to demonstrate –SH groups in the stratum corneum but Hardy (*9*), Mescon and Flesch (*22*), and Barrnett (*2*) found some reaction in it.

Although the keratohyalin granules in the stratum granulosum are considered the seat of abundant –SH groups (*7, 9*), we find, in agreement

with Barrnett (*2*), that they are unreactive in all the material observed but that the ground cytoplasm of these cells is strongly reactive.

Giroud and Bulliard (*3–5*) and Giroud and Leblond (*6*), largely on the basis of staining reactions with nitroprusside, concluded that two types of keratinization occur in the skin—"soft" and "hard." "Soft" keratinization occurs in the surface epidermis where the Malpighian layer stains homogeneously and the stratum corneum not at all. "Hard" keratinization occurs in the hair cortex, where the intense reaction in the "keratogenous zone" ends abruptly in the keratinized part of the hair shaft. In addition, these authors (*6*) find that soft keratin contains larger amounts of lipid than hard keratin, and hard keratin is richer in sulfur. Although different keratins exist, we believe, in agreement with Chèvremont and Frederic (*7*), Lapiere (*23*), Hardy (*9*), Mescon and Flesch (*22*), and Barrnett (*2*) that one cannot designate "soft" and "hard" keratinization on the basis of reactions for –SH groups. We often find in the surface epidermis, at the limit between the Malpighian and corneal layers, a strong reaction that is comparable to the "keratogenous zone" in the hair follicle. In the tongue and esophagus, as well as in the epithelioma of the hairless mouse, the keratinized layer is as intensely reactive as the "keratogenous zone" of hair follicles. In all of these cases the cornified layer consists of partially keratinized cells all of which still show a moderately distinct nucleus. This condition, which occurs normally in the stratified squamous epithelium of mucous membranes, is called parakeratosis. In the "keratogenous zone" of the hair root, the cells are incompletely keratinized, show a distinct nucleus, and must be considered parakeratotic. Similarly, the cells that surround the radicles of the hair club in resting hair follicles are parakeratotic. All of these parakeratotic regions give an intense reaction for –SH groups. This reaction, then, seems to have little to do with hard and soft keratinization. As Chèvremont and Frederic (*7*) have said, ". . . il y a seulement des différences de degrés entre les divers processus morphologiques de la kératinisation."

Summary

1) In the mouse, skin with telogen hair follicles shows abundant –SH groups only around the brush of the hair club. During early anagen, there is an increase in –SH groups in the thickened epidermis, and in the external sheath of the developing hair follicle. Through anagen VI, the greatest concentration of –SH groups is in the cortex and its cuticle in the "keratogenous zone" of the hair shaft. Above this region, the mature cortex and cuticle are nonreactive. The internal sheath and medulla also contain some –SH groups. During catagen, the brush of the keratinizing hair club contains abundant –SH groups. The rest of the cortex shows no demonstrable –SH groups. The external and internal sheaths show a higher concentration of –SH groups than they did during anagen.

2) In the hair follicles of the guinea pig skin, the localization of –SH groups is essentially similar to that found in the mouse.

3) The stratum corneum in the skin of the mouse and guinea pig always shows the presence of some –SH groups.

4) In the stratum granulosum, in all the material observed, the keratohyalin granules are unreactive to the –SH reagent.

5) The Malpighian layers of the esophagus, tongue, and sole pads of normal mice, and of an epithelioma from a hairless mouse, contain strong concentrations of –SH groups. The parakeratotic layer that covers all of these tissues shows a particularly intense reaction.

6) It is concluded that protein-bound –SH groups are revealed in greatest abundance in tissues undergoing keratinization and particularly in parakeratotic regions. The "keratogenous zone" in hair follicles is the parakeratotic region of the hair root.

7) "Soft" and "hard" keratinizations as defined by Giroud and Bulliard do not take place.

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PLATE 27

These figures represent 10 μ sections of skin of mice treated with the sulfhydryl reagent of Barrnett and Seligman.

FIGURE 1.—Hair follicle in telogen showing an intense reaction around the hair club. The cortex immediately above this region is unstained. Arrow points to the unreactive dermal papilla. *Ca.* × 270

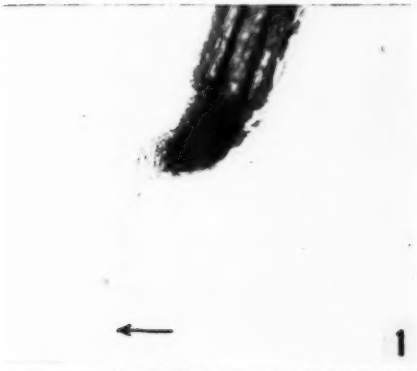
FIGURE 2.—Hair follicle in anagen II. The cells in the thickened epidermis as well as the external sheath of the developing hair follicles show abundant –SH groups. *Ca.* × 270

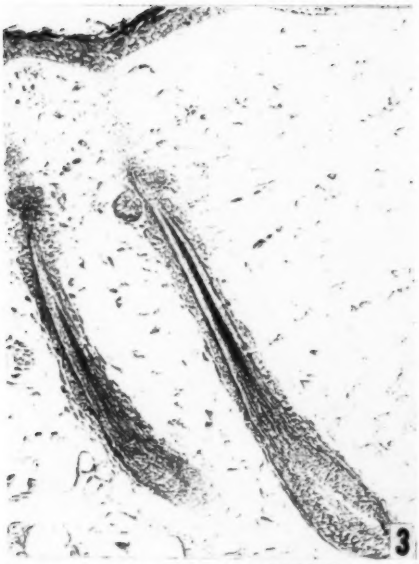
FIGURE 3.—Hair follicle in anagen IV. The tip of the hair, which is composed only of cuticle and cortex, is in the "keratogenous zone," and exhibits an intense reaction while the rest of the follicle stains faintly. *Ca.* × 150

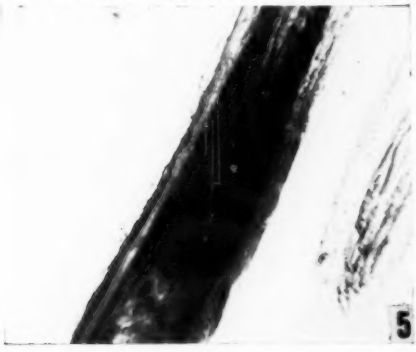
FIGURE 4.—Hair follicle in anagen VI, through the "keratogenous zone" of the hair root. The reaction disappears abruptly, just above this region. *Ca.* × 150

FIGURE 5.—Detail of the "keratogenous zone" of a follicle in anagen VI. *Ca.* × 270

FIGURE 6.—Hair follicle in early catagen. Intense reaction only in the cortex in the region of the developing club. The arrow points to the dermal papilla, which is unstained. *Ca.* × 270







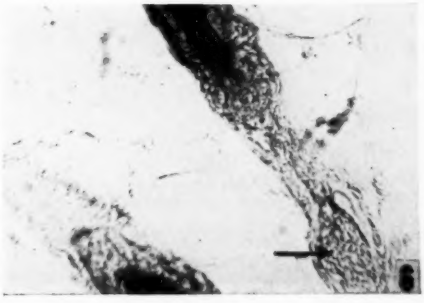


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PLATE 28

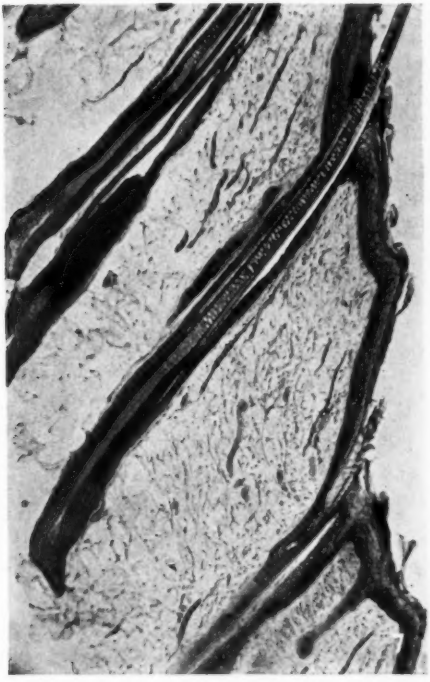
These figures represent 10 μ sections of the skin of guinea pigs treated with the sulfhydryl reagent.

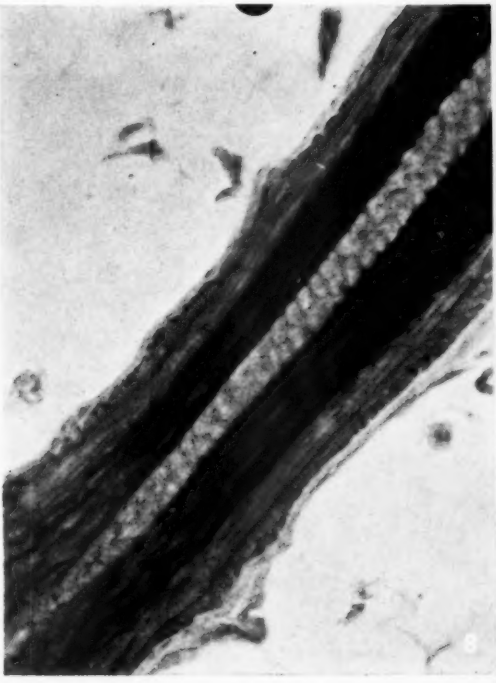
FIGURE 7.—Hair follicle in late anagen. The "keratogenous zone" is stained intensely; the reaction ends suddenly just above this zone. The medulla of the hair contains –SH groups in its entire length. *Ca.* × 60

FIGURE 8.—Enlarged detail of figure 7 showing the lower portion of the "keratogenous zone"; observe the fibrillar nature of the hair cortex in this region. The external sheath, Henle's layer of the internal sheath, and the medulla are also reactive. *Ca.* × 270

FIGURE 9.—Enlarged detail of the upper part of the "keratogenous zone" in the same follicle. The very strong –SH group reaction in the cortex ends abruptly and the rest of the cortex is unstained. The medulla of the hair shows a moderate reaction. Note that the cuticle of the internal sheath stains intensely. *Ca.* × 270

FIGURE 10.—Hair follicle in catagen. The cortex of the hair is unstained but the brushlike projections of the club are surrounded by a dark –SH reactive band. The external sheath shows a moderately intense reaction. *Ca.* × 270





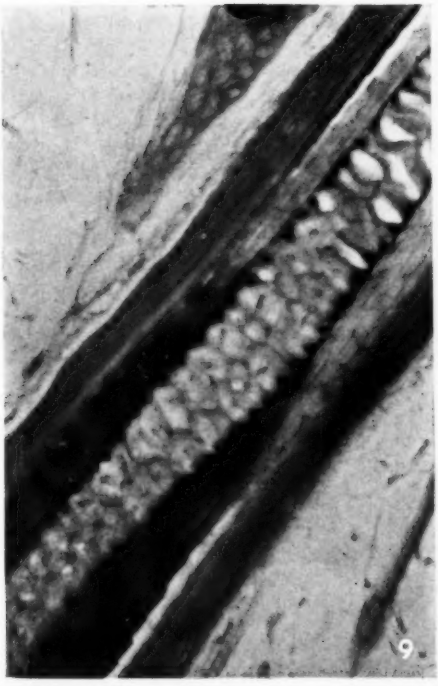


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PLATE 29

FIGURE 11.—Epithelioma from a hairless mouse. The thick Malpighian layer, and particularly the deep parakeratotic layer, stains very intensely. The parakeratotic tips of the papillomatous projections show the most intense reaction. *Ca.* × 60

FIGURE 12.—Sole pad of the mouse. The Malpighian layer and the stratum corneum stain strongly with the –SH reagent. Note the intense reaction around the luminal borders of the small sweat glands. *Ca.* × 60

FIGURE 13.—Enlarged detail of the same sole pad to show the strong –SH group reaction in the epidermis and stratum corneum. The arrow points to the stratum granulosum, where the keratohyalin granules are not reactive. *Ca.* × 562.5

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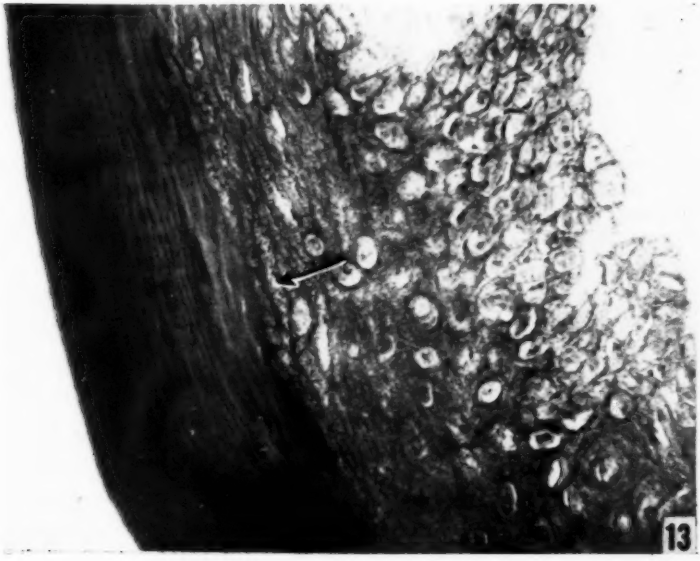
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The Nature and Staining Reactions of the Fibroma-Cell Inclusions of the Shope Fibroma of the Rabbit [1]

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In the course of study of the Shope fibroma of the rabbit, it became apparent that little had been written concerning the intracytoplasmic inclusions found in the fibroma cells of these lesions. It will be recalled that in the development of the Shope fibroma there is an initial inflammatory stage. Usually after one week to ten days this is followed by the formation of a tumor comprised of large polygonal and round cells within the corium. These cells have abundant acidophilic cytoplasm (shown by staining with hematoxylin and eosin) and vesicular nuclei. The latter demonstrate degenerative changes as the lesion progresses. By the use of Mallory's phosphotungstic acid hematoxylin procedure fibroglia are occasionally seen arising from the tumor cells. There is little collagen produced however, and frequently the tumor cells lie in an edematous matrix. Within the cytoplasm of the tumor cells are granules and homogeneous bodies varying from the size of micrococci to that of globules, in the later stages, filling the entire cytoplasm. They do not display any particular distribution pattern or geometric design. These inclusions are not clearly visible with the routine hematoxylin and eosin staining method, but appear a distinct blue when sections are stained by Heidenhain's azan procedure. However, when the bodies are small or scarce, oil immersion magnification must be used for clear demonstration. With the periodic acid-Schiff procedure the inclusions are strongly positive. This method makes the bodies clearly visible at a magnification of 450 to 600, thus making them more conspicuous than with previously reported methods.

There have been few reports on the detailed morphology of the Shope fibroma, and in these the nature of the intracytoplasmic inclusion bodies has been merely speculated on. Shope [(*1*) 1932] in his original article failed to mention the presence of these bodies, although he did note the epidermal inclusions that resemble those of molluscum contagiosum. Hyde [(*2*) 1936], utilizing methyl alcohol fixation and the Giemsa-Wolbach stain on wet preparations, saw bipolar red-staining bodies within the bluish cyto-

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[2] The author is grateful to Mr. Joseph Woodard for his valuable technical assistance.
[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

plasm of the tumor cells and suggested that they represented the etiologic virus or aggregates of virus. Hurst [(*3*) 1937], however, reported that the granules were azurophilic with Giemsa's stain, although some were oxyphilic, and in addition he noted that they demonstrated a faint Feulgen reaction. Ahlström [(*4*) 1938] observed that the inclusions were clearly demonstrated with Heidenhain's azan procedure, which stained them blue. He noted also that they were stained positively by Weigert's methyl violet procedure for fibrin and had a red-violet color with Mallory's phosphotungstic acid hematoxylin stain. He was of the opinion that the "basophilic" inclusions in the fibroma cell could be identified as protein in nature, and although he considered the possibilities that they represented either virus particles, degenerated products of the cell cytoplasm, or phagocytized material from the surrounding medium, he favored the last-mentioned.

The purpose of this paper is to report the results of further investigation of the nature of these intracytoplasmic inclusion bodies, utilizing not only many classical staining procedures of histologic investigation but also some of the more recent histochemical methods.

Materials, Methods, and Results

Blocks of Shope fibroma of the rabbit, supplied by Dr. Lawrence Kilham,[4] were fixed in 10% formalin, 95% alcohol, and Zenker's acetic fluid. All were dehydrated and infiltrated with paraffin, and mounted with water or alcohol according to routine methods. In addition, blocks were fixed in 65% alcohol and prepared for alkaline phosphatase determinations according to the method of Mowry (*5*). The staining procedures and histochemical methods were performed as described in Lillie's *Histopathologic Technic* (*6*) unless otherwise designated.

I. General oversight methods:

A. Alum hematoxylin and eosin——not visualized.
B. Iron hematoxylin and eosin——not visualized.
C. Azure-eosin——not visualized.
D. Thionin, 0.05% in M/100 acetate buffer, *p*H 4 and *p*H 7 for 30 min.——not visualized.
E. Methyl blue-eosin (Mann's stain)——blue.

II. Collagen methods:

A. Masson trichrome with aniline blue——pale blue.
B. Heidenhain azan modification of Mallory——dark blue.
C. Deamination with HNO_2 (6 gm. $NaNO_2$, 5 cc. glacial acetic acid, water qs. ad. 40 cc.) for 24 hours at 25° C. followed by Heidenhain azan method——not visualized (effective deamination).
D. Mallory's phosphotungstic acid hematoxylin——yellow-orange (lighter than collagen).

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E. Van Gieson (Unna)——not visualized.
F. Van Gieson (Unna) with picrofuchsin for one hour at 60° C.——red-brown.
G. Fast green van Gieson——not visualized.
H. Wilder's method for reticulum——light brown (slight decoloration of silver).

III. Elastic tissue methods:
A. Orcein——not visualized.
B. Weigert's stain for elastic fibers——not visualized.

IV. Fibrin and bacterial methods:
A. Gram-Weigert——not visualized.
B. Brown-Brenn (*7*) method for bacteria——not visualized.
C. Ziehl-Neelsen——not visualized.

V. Amyloid methods:
A. Congo red——not visualized.
B. Crystal violet——not visualized.

VI. Lipid methods:
A. Oil red O on frozen sections——not visualized.
B. Sudan black B at 60° C. on frozen and paraffin sections——not visualized.
C. Frozen and paraffin sections placed in pyridine at 60° C. for 24 hours followed by periodic acid-Schiff method——red-purple (unaffected).
D. Peracetic acid-Schiff method for demonstration of unsaturated compounds——not visualized.

VII. Mucin methods:
A. Mucihematin——not visualized.
B. Mucicarmine——not visualized.
C. Rinehart, Abul-Haj (*8*) method for acid mucopolysaccharides——not visualized.

VIII. Periodic acid-Schiff methods:
A. Periodic acid-Schiff alum hematoxylin——red-purple.
B. Barley malt diastase 0.1% in saline phosphate buffer of *p*H 6, 1 hour at 37° C. followed by periodic acid-Schiff method for demonstration of glycogen. Glycogen-laden liver sections as activity control——red-purple (unaffected).
C. Hydroxyl and amine blockade by acetylation with acetic anhydride : pyridine (16:24) for 24 hours at 25° C. followed by periodic acid-Schiff method——not visualized (effective blockade).
D. Blockade of carboxyl and other acid groups by methylation with 0.05 N HCl in methanol for 24 hours at 60° C. and also for 1 week at 25° C. followed by periodic acid-Schiff. Controls in plain methanol under similar conditions red-purple (unaffected).

E. Allochrome procedure of Lillie——red-purple.
F. Direct Schiff reaction for 72 hours——not visualized.
G. Pretreatment with hyaluronidase (Wyeth lot #173A1, 1500 TRU/mg.) 150 units/cc. in *p*H 5.5 sodium acetate-acetic acid buffer for 1 hour at 37° C. followed by periodic acid-Schiff. Control sections of cock's comb similarly pretreated and followed by Rinehart, Abul-Haj method——red-purple (unaffected).
H. Digestion of sections with trypsin (0.1% 4X U.S.P. Pancreatin) in M/100 phosphate buffer, *p*H 7.6 for 1, 2, and 3 hours at 37° C. followed by periodic acid-Schiff. Control sections treated with buffer alone——not visualized after 2 hours. Control section unaffected.
I. Digestion of sections with pepsin (0.1% Control #401003, Difco Lab.) dissolved in N/10 HCl. Control sections in plain N/10 HCl. Sections incubated at 37° C. for 2, 6, 12, 24, and 28 hours followed by periodic acid-Schiff——red-purple (unaffected).
J. Digestion with ribonuclease (crystalline ribonuclease 1:10,000 Armour lot #7095X in *p*H 6 phosphate buffer for 1 hour at 37° C. followed by periodic acid-Schiff. Activity control, section of dog pancreas——red-purple (unaffected).

IX. Other methods:

A. Feulgen nucleal procedure for desoxyribonucleic acid (1 N HCl hydrolysis for 5, 10, and 20 minutes)——not visualized.
B. Diazo-safranin method of Lillie and Burtner (*9*), a coupling reaction for phenols——not visualized.
C. Gomori's methenamine silver method. Burtner-Lillie variant (*10*) for demonstrating di- or polyhydroxy- or NH_2OH aryl compounds ——not visualized.
D. Ferric ferricyanide reduction test (*11*) for the demonstration of sulfhydryls, phenols, etc.——not visualized.
E. Ninhydrin-Schiff method (*12*), a proposed test for demonstration of protein——red-purple (positive).
F. Millon reaction for tyrosine-containing protein——orange-brown (faint positive).
G. Alkaline phosphatase method of Gomori——not visualized.
H. Feulgen-Voigt plasmal reaction on frozen sections for acetal phosphatides, Hayes variant (*13*)——not visualized.
I. Winkler-Schultze method for peroxidases. Control section of ceroid liver——not visualized.

X. Birefringence——none.

XI. Fixation——The intracytoplasmic inclusions are similar in their staining reactions following alcohol, Zenker acetic, or formalin fixation, except that the periodic acid-Schiff reaction on formalin-fixed tissue is less intense than with the other fixatives used. Tryptic digestion is observed only after alcohol fixation.

Although Ahlström referred to the fibroma-cell inclusions as being "basophilic," it seems evident that he was referring to their staining blue with the strongly acid dye, aniline blue, as used in the Mallory-Heidenhain azan method. We have been unable to stain these bodies with basic dyes following formalin, Zenker acetic, or alcohol fixation, and in addition they are colored by methyl blue, a trisulfonic acid dye in the Mann procedure. One must conclude that these bodies are cyanophilic but not truly basophilic. The Feulgen nucleal reaction has been consistently negative, and digestion with ribonuclease has failed to alter other staining reactions that color these inclusions. It follows that desoxyribonucleic or ribonucleic acids are not present within the inclusions and it is therefore most unlikely that they represent the etiologic virus, since viruses are purported to possess nucleoprotein structure.

Histochemically there is evidence that the intracytoplasmic inclusions contain protein other than nucleoprotein. They are promptly digested by trypsin, faintly positive with Millon's reagent, and strongly positive following the ninhydrin-Schiff method of Yasuma and Ichikawa (a recent technique proposed for the detection of protein). Inhibition of the blue color produced by the azan reaction with nitrous acid deamination indicates the presence of amine groups, most probably in a protein moeity. Indirect evidence for their protein nature is added when it is noted that tryptic digestion occurs only after alcohol fixation but not when tissues have been fixed in Zenker's acetic acid or formalin. This indicates that the chemical structure of the substrate has been altered by the latter two fixatives. The tanning (crosslinking) reactions of formalin and chromic salts with protein are well known.

Because of the protein nature of these bodies, as well as the observation that there is fragmentation of pre-existing collagen with the advancing growth of the tumor, the possibility that these inclusions represent phagocytized collagen presents itself. However, the fibroma cells are not phagocytic for trypan blue according to Hyde (*2*). Aside from the positive azan and Mann procedures, other collagen stains are negative, and in both the positive aniline blue or methyl blue methods exposure of the dye is either greatly prolonged or practiced at a higher temperature than that necessary to stain collagen. The lack of birefringence and absence of peptic digestion also indicate that the fibroma-cell inclusions are dissimilar to collagen.

The strongly positive periodic acid-Schiff reaction of the fibroma-cell inclusions indicates that another substance other than protein is present, since the reaction is more intense than that observed with protein substances. That the reactive material is not glycogen is evident from the lack of diastase digestion. The failure to stain these bodies with methods for mucin and acid mucopolysaccharides and the lack of hyaluronidase digestion eliminates these possibilities. Aside from acetylation, other procedures utilized to investigate the nature of the periodic acid-Schiff-positive material are of little help in this instance. The effective blockade

of the periodic acid-Schiff staining produced by acetylation does indicate the presence of 1,2 glycols or hydroxy amine groups.

From the results obtained one may state that the fibroma-cell inclusions of the Shope fibroma of the rabbit are conjugated protein in nature, probably glycoprotein. It seems most likely that they represent a product of cytoplasmic activity, rather than the etiologic virus or phagocytized material from the surrounding environment.

Conclusions

The fibroma-cell inclusions of the Shope fibroma of the rabbit are intensely colored by the periodic acid-Schiff method. This allows them to be more easily recognized in tissue sections than was possible with previously reported staining procedures.

From the results of this study, using various histochemical and histologic methods, it is concluded that the intracytoplasmic inclusions are chemically conjugated protein, most probably glycoprotein, and that they represent a product of cytoplasmic activity rather than the etiologic virus or phagocytized material.

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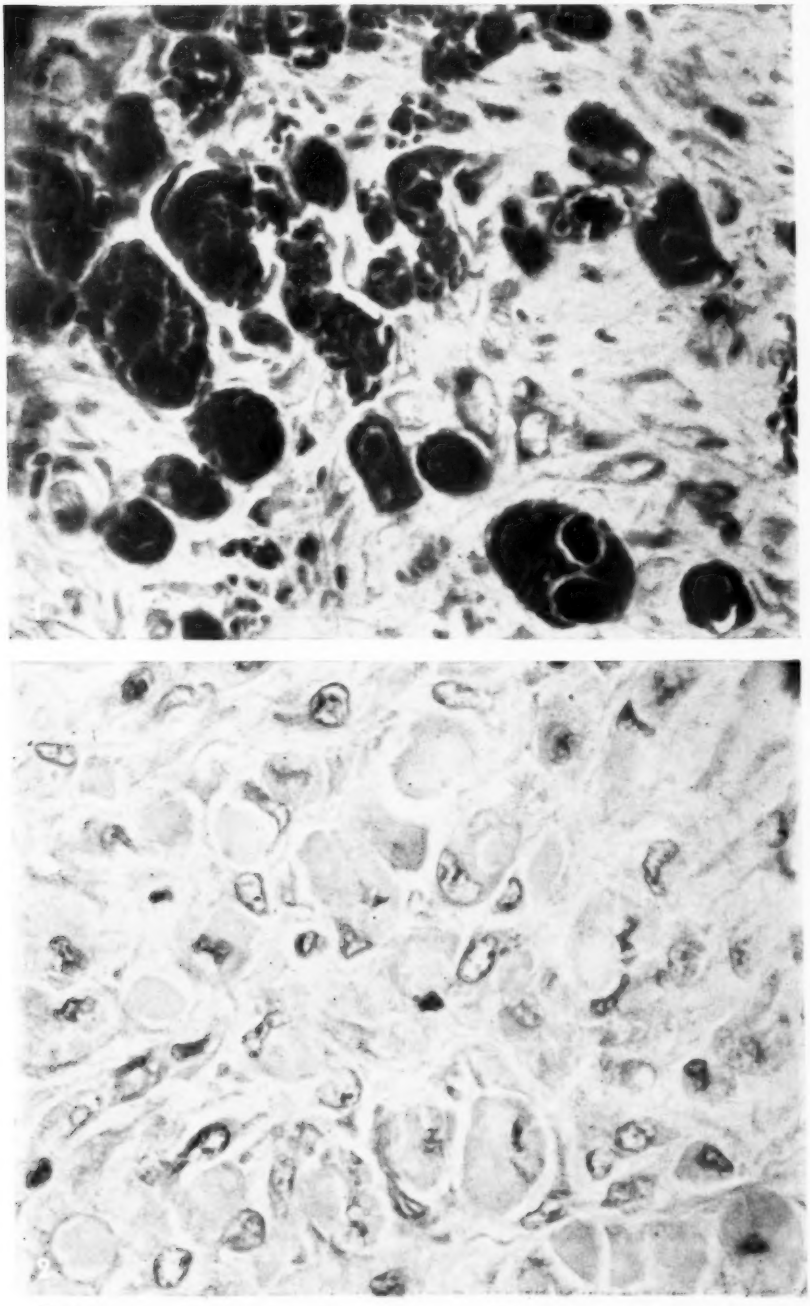
PLATE

PLATE 30

FIGURE 1.—Periodic acid-Schiff method demonstrating strongly positive intracytoplasmic inclusions. × 615

FIGURE 2.—The inclusion bodies are not distinct (compare with figure 1). Hematoxylin and eosin. × 615



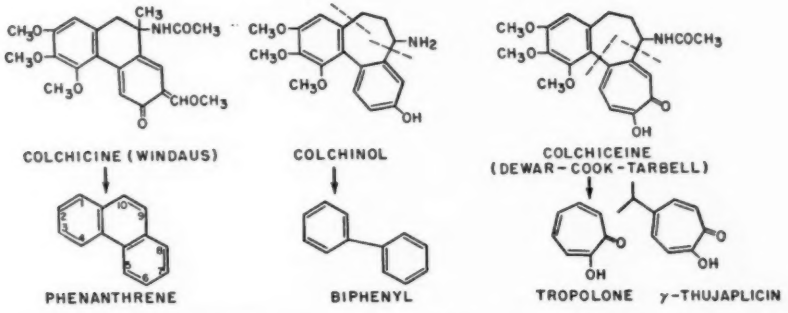
Fisher

Damage Induced in Sarcoma 37 with Chemical Agents. VI. Biphenyl, Fluorene, Phenanthrene, and Tropolone Derivatives [1]

J. Leiter, J. L. Hartwell, J. S. Kahler, I. Kline, *and* M. J. Shear, *Laboratory of Chemical Pharmacology, National Cancer Institute,*[2] *Bethesda, Md.*

Investigations in several series of colchicine derivatives (*1–3*), of the relationship between chemical structure and potency in damaging Sarcoma 37, revealed marked differences among these compounds both in the tumor-damaging potency and in the dose required for lethality following a single subcutaneous injection into mice bearing six-day-old tumor implants. For most of these derivatives, the changes in structure represented differences in substituents on the basic three-ring structure of colchicine, or in changes in the nature of the C ring, *viz.*, degradation to a six-membered aromatic ring (colchinol series), or reduction of the C ring (hexahydrocolchicine).

In addition to these types of derivatives, we examined compounds with more distant structural relationships to colchicine. They included types of compounds that would result when one or another of the three rings was opened, or when one or more of the rings is eliminated. Thus by appropriate splitting of ring B (text-fig. 1) in the colchinol or colchicine



Text-figure 1.—Relationship of biphenyl and tropolone to colchinol and colchicine.

[1] Received for publication May 21, 1953.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

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molecule, compounds related to biphenyl or to seven-membered ring compounds similar to tropolone could be obtained.

In addition to these analogs, a small number of phenanthrene derivatives and related heterocyclic compounds were examined. Interest in such compounds arose from the earlier formulation of colchicine as a reduced phenanthrene derivative (*4*).

This paper reports the effects of a single subcutaneous injection of such compounds in producing damage in Sarcoma 37.

Materials and Methods

The compounds employed were obtained from the sources listed below. In the accompanying tables, the source of each compound is indicated by the following code letters:

Code	*Source*
A.....	Dr. A. B. Anderson, Oregon Lumber Co., Dee, Oreg.
B.....	Bios Laboratories, New York, N. Y.
C.....	Dr. Kenneth N. Campbell, Dept. of Chemistry, Univ. of Notre Dame, Notre Dame, Ind.
D.....	Delta Chemical Works, New York, N Y.
E.....	Eastman Kodak Co., Rochester, N. Y.
F.....	Dr. W. S. Johnson, Univ. of Wisconsin, Madison, Wis.
G.....	A. D. Mackay, New York, N. Y.
H.....	Merck and Co., Rahway, N. J.
I.....	Dr. Erich Mosettig, National Institutes of Health, Bethesda, Md.
J.....	National Aniline Div., Buffalo, N. Y.
K.....	Dr. P. L. Pauson, Univ. of Sheffield, England
L.....	Dr. L. J. Sargent, National Institutes of Health, Bethesda, Md.
M.....	Dr. D. S. Tarbell, Dept. of Chem., Univ. of Rochester, Rochester, N. Y.
N.....	Fränkel and Landau, Germany
O.....	Dr. Jarl Gripenberg, Finland Institute of Technology, Helsingfors, Finland
P.....	Dr. Lyndon F. Small, National Institutes of Health, Bethesda, Md.
Q.....	G. D. Searle & Co., Chicago, Ill.
R.....	L. A. Pinck, Dept. of Agriculture, Beltsville, Md.
S.....	Edcan Laboratories, So. Norwalk, Conn.
T.....	Farchan Research Laboratories, Cleveland, Ohio
U.....	L. Light & Co., Ltd., Colnbrook, Bucks, England

The tumor employed was Sarcoma 37; the mice were CAF_1 hybrids. The methods for evaluating damage induced in the tumors were similar to those described previously (*1–3*).

Results

Biphenyls.—Of a series of 15 biphenyl compounds injected into mice bearing six-day-old tumor implants, all but one were inactive in producing damage in the tumors. Table 1 summarizes the data with respect to the highest dose given, the vehicle employed, and the number of animals that died during the observation period (usually 24 hours.) Of particular inter-

TABLE 1.—*Biphenyl derivatives inactive against Sarcoma 37, following a single subcutaneous injection of a maximum tolerated dose*

NCI No.	Compound	Source	Highest dose given (μg./gm.)	Mortality*
331	Tetramethylbenzidine †	E	1, 000	1/15
369	2′-Amino-4′,2,3,4-tetramethoxybiphenyl †	M	500	0/8
411	*o*-Diethylaminobiphenyl†	E	800	0/8
412	*p*-Diethylaminobiphenyl †	E	1, 000	0/8
944	*o*-Iodobiphenyl ‡	E	1, 000	0/8
945	*p*-Iodobiphenyl‡	E	1, 000	0/8
1547	4-(3,4,5-Trimethoxyphenyl)-1-cyclohexene-5-carboxylic acid †	M	500	0/8
1548	1,2-Dimethyl-4-(3,4,5-trimethoxyphenyl)-1-cyclohexene-5-carboxylic acid †	M	500	0/8
1937	3-Methyl-5-(3,4-dimethoxyphenyl)-6-ethoxymethylene-2-cyclohexen-1-one‡	C	1, 000	0/8
1938	3-Methyl-5-(3,4-methylenedioxyphenyl)-6-ethoxymethylene-2-cyclohexen-1-one‡	C	1, 000	2/15
1939	3-Methyl-5-(2,3,4-trimethoxyphenyl)-6-ethoxymethylene-2-cyclohexen-1-one‡	C	1, 000	0/15
3202	Benzidine HCl†	E	300	2/8
3226	3-(1′,2′,3′-Trimethoxyphenyl)-5-methylphenol†	C	1, 000	0/8
335	α,α′-Dipyridyl†	E	250	4/15 §
			200	0/23 §
1866	α,α′,α″-Dipyridyl-tripyridyl †	B	0. 25	0/15 ‖

*Expressed as: $\frac{\text{Number of mice dead}}{\text{Number of mice treated}}$.

†In aqueous solution of dilute acid or dilute alkali.

‡In olive oil as vehicle.

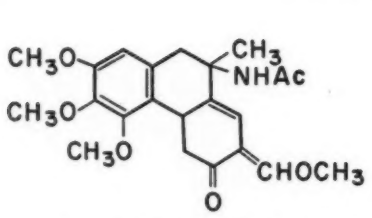
§Damage induced in 6 of 11 survivors at 250 μg. dose and in 12 of 23 survivors at 200 μg. dose. No damage was induced at 150 μg./gm.

‖The high toxicity of this compound is noteworthy. All of the 23 mice injected with a dose of 0.5 μg./gm. were killed. All of 4 non-tumor-bearing mice injected with this dose were likewise killed.

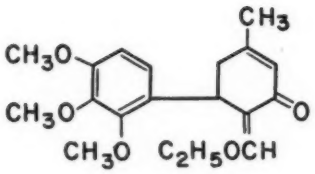
est is the lack of potency of 2′-amino-4′,2,3,4-tetramethoxybiphenyl (NCI-369), which is similar to a product that would be obtained when the B ring of the methyl ether of colchinol (text-fig. 1) is opened; it was inactive even at a dose (500 μg./gm.) approximately 4 times the MED (minimum effective dose) of the parent colchinol compound (125 μg./gm.). Of interest, too, are compounds NCI-1937, 1938, and 1939, which contain the functional ketone and hydroxymethylene groups of the Windaus formulation of colchicine (see text-fig. 2); these compounds were all inactive even at a dose of 1,000 micrograms per gram.

The only compound in this series that damaged Sarcoma 37 was α,α′-dipyridyl (NCI-335) (see footnote §, table 1, and text-fig. 2). This compound is an analog of biphenyl, having two pyridine rings instead of two benzene rings. A dose of 200 μg. per gm., near the maximum tolerated (about 250 μg. per gm.), was required to induce damage in the tumors.

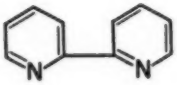
About 20 other biphenyl compounds, quaternary ammonium or sulfonium derivatives, were also examined. A few of them had potency in damaging Sarcoma 37. Since the behavior of these compounds appeared to be more related to their quaternary properties than to the biphenyl component, they will be reported later together with other quaternized compounds.



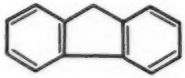
COLCHICINE (WINDAUS)



NCI-1939



α,α'-DIPYRIDYL (NCI-335)



FLUORENE

TEXT-FIGURE 2.

Because of the close formal relationship of fluorene compounds to biphenyls (see text-fig. 2), the 15 we examined are included in this paper (table 2). All were inactive at tolerated doses; however, 9-aminofluorene (NCI-800) and 9-methyl-9-aminofluorene (NCI-801) induced damage in the tumors at doses well above the MTD (see footnotes ‡ and §, table 2).

TABLE 2.—*Fluorene derivatives negative against Sarcoma 37 following a single subcutaneous injection of a maximum tolerated dose*

NCI No.	Compound (*in olive-oil vehicle*)	Source	Highest dose given (*μg./gm.*)	Mortality*
32v	Fluorenone	E	1,000	0/15
635	2-(3-Diamylamino-1-hydroxypropyl)-fluorene ·HCl.	P	1,000	0/8
700	9-Hydroxy-9-carboxyfluorene†	J	1,000	0/8
73v	Pavatrine†	Q	700	4/8
799	1-Aminodibiphenylene-ethane	R	1,000	0/15
80v	1-Methylaminodibiphenylene-ethane	R	1,000	0/8
80x	1-Ethylaminodibiphenylene-ethane	R	1,000	0/8
800	9-Aminofluorene·HCl	R	800	6/10‡
801	9-Methyl-9-aminofluorene·HCl†	R	75	6/8§
802	9-Phenyl-9-aminofluorene	R	1,000	0/7
803	9-(α-Naphthyl)-9-aminofluorene	R	1,000	0/8
858	Difluorenyl	S	1,000	0/8
1036	2,4,7-Trinitrofluorenone	T	1,000	0/8
1464	2-Nitrofluorene	E	1,000	0/8
2319	2-Aminofluorene	U	800	7/16

*Expressed as: $\frac{\text{Number of mice dead}}{\text{Number of mice injected}}$.

†In aqueous vehicle.

‡At 500 μg./gm. 12 of 15 mice died; the tumors in the 3 survivors were negative.
" 600 " " 10 of 10 " " ; no survivors.
" 800 " " 6 of 10 " " ; tumor damage was induced in all 4 survivors.

§At 100 μg./gm. 7 of 8 " " ; the survivor showed induced tumor damage.
" 75 " " 6 of 8 " " ; 1 of the 2 survivors showed induced tumor damage.

TABLE 3.—*Damage induced in Sarcoma 37 following a single subcutaneous injection of tropolones and related seven-membered ring compounds*

NCI No.	Compound	Source	Vehicle	Dose (μg./gm.)	Mortality*	Induced damage after 24 hrs.†
3065	*o*-Bromotropolone	C	Olive oil	200	4/8	2/4
			" "	150	1/8	0/7
2390	γ-Thujaplicin (*p*- or 5-isopropyltropolone)	A, O	Aqueous	500	5/9‡	2/4
			"	400	1/10‡	5/9
			"	300	0/5	0/5
2469	4,5-Benztropolone	M	Olive oil	1, 000	0/8	0/8
2430	4,5-Benztropolone methyl ether	M	" "	200	5/8	0/3
			" "	150	0/8	0/8
2281	Purpurogallin (2′,3′,4′-trihydroxy-3,4-benztropolone)	D	Aqueous	500	1/15	0/14§
2625	Purpurogallin tetramethyl ether	K	Olive oil	1, 000	0/8	0/8
3066	3,3,7-Trimethylcyclohepta-4,6-diene-2-ol-1-one	C	Aqueous	1, 000	1/8	0/7
2811	Cycloheptanone	C	Olive oil	1, 000	2/8	0/6
2812	1,2-Cycloheptanedione	C	" "	600	0/16‡	6/16
			" "	400	0/13‡	2/13
3067	3,3,7-Trimethylcyclohepta-4,6-diene-1,2-dione	C	" "	1, 000	2/5	0/3
			" "	800	0/5	0/5
2738	β-(1-Hydroxycycloheptyl)-acrylic acid lactone	C	" "	200	2/8	0/6

*Expressed as: $\frac{\text{Number of mice dead}}{\text{Number of mice treated}}$.

†Expressed as: $\frac{\text{Number of mice with induced damage}}{\text{Number of mice sacrificed}}$. The tumors in untreated control mice showed no induced damage.

‡Results of 2 separate experiments.

§Some of these represent animals sacrificed at 8- and 48-hour intervals.

Tropolones.—Table 3 summarizes the effects obtained following a single subcutaneous injection of tropolone derivatives and related seven-membered ring compounds. Several systems of nomenclature are used for the positions on the ring. In all cases, orientation is figured from the ketone position. Other positions are identified as α-, β-, γ-, or *o*-, *m*-, *p*-, or 3-, 4-, 5-, respectively.

Of the tropolone derivatives, only the simple tropolones (NCI–3065 and 2390) produced damage in the tumors, but a dose at or above the MTD was required. None of the tropolones having a fused benzene ring (NCI–2469, 2430, 2281, and 2625) showed any activity. Cycloheptanedione (NCI–2812) was the only other compound in this group producing damage in the tumors. The activity of this compound appeared to be of a low order, although it had low toxicity—only one death was obtained with a dose of 1,000 μg. per gm.; damage was induced in some of the tumors with a dose of 400 or 600 μg. per gm.

Phenanthrenes and related compounds.—The only compounds related to phenanthrene that produced damage in Sarcoma 37 are listed in table 4; the negative compounds are listed in table 5 with the highest dose used and the mortality. Of the five positive compounds listed in table 4, the first two (NCI-913 and 701) are true phenanthrene derivatives. The former, however, has a biguanide functional group and the latter a quinone group. The last three compounds are analogs of phenanthrene in which two heterocyclic rings replace benzene rings. Of these, only *o*-phenanthroline (NCI-92v) was able to damage Sarcoma 37 at a dose (30 μg./gm.)

TABLE 4.—*Damage induced in Sarcoma 37 with a single injection of phenanthrene derivatives or related compounds*

NCI No.	Compound (*in aqueous vehicle*)	Source	Dose (μg./gm.)	Mortality *	Induced damage after 24 hrs.†
913	1-Methyl-5-(9-phenanthryl)-biguanide·HCl.	I	‡ 300	2/16	8/13
			‡ 200	0/16	10/16
			‡ 100	0/16	2/16
701	Phenanthraquinone §.....	J	‡ 1, 000	0/13	3/13
92v	*o*-Phenanthroline.....	G	100	3/8	5/5
			‡ 80	1/16	8/15
			40	0/8	7/8
			30	0/16	6/16
1275	*m*-Phenanthroline·$2H_2O$.....	B	180	6/8	2/2
			150	2/8	0/6
1276	*p*-Phenanthroline·H_2O.....	B	‡ 180	2/16	12/14
			‡ 120	0/16	10/16
			100	0/8	0/8

*Expressed as: $\frac{\text{Number of mice dead}}{\text{Number of mice treated}}$.

†Expressed as: $\frac{\text{Number of mice with induced damage}}{\text{Number of mice sacrificed}}$. The tumors in untreated control mice showed no induced damage.

‡Two separate experiments.

§Olive-oil vehicle.

appreciably below the maximum tolerated (80–100 μg./gm.). As with the biphenyl derivatives, the quaternary compounds containing phenanthrene as a substituent are not reported at this time.

TABLE 5.—*Phenanthrene derivatives negative against Sarcoma 37 following a single subcutaneous injection*

NCI No.	Compound	Source	Highest dose given (μg./gm.)	Mortality*
	Substituted Phenanthrene (olive-oil vehicle)			
126	9-Amino-	I	600	0/16
127	9-Acetylamino-	I	1, 000	0/8
128	9-Diacetylamino-3-methoxy-	I	1, 000	0/15
129	9-Acetylamino-10-hydroxy-	I	1, 000	0/8
13v	9-Acetylamino-10-methoxy-	I	1, 000	0/7
13x	9-Acetylamino-10-ethoxy-	I	1, 000	0/6
1055	9-Aminomethyl-·HCl	I	1, 000	0/8
1056	9-(2-Aminoethyl)-·HCl	I	1, 000	0/16
1057	9-Aldehyde-	I	1, 000	0/8
911	1-(9-Phenanthryl)-biguanide·HCl†	I	200	1/8
912	1,1-Diethyl-3-(9-phenanthryl)-guanidine·HCl†	I	60	7/8
915	1-Isopropyl-5-(3-phenanthryl)-biguanide·HCl	I	1, 000	0/8
914	2-Methyl-2-thio-3-cyano-1-(9-phenanthryl)-pseudourea.	I	1, 000	0/8
1691	3,4-Dimethoxy-6-keto-13-ethyloctahydrophenanthrene.	L	1, 000	0/8
	Phenanthraquinones and α,β-unsaturated ketones			
645	1-Keto-2-hydroxymethylene-1,2,3,4-tetrahydrophenanthrene‡.	F	840	0/15
646	1-Keto-2-hydroxymethylene-7-methoxy-1,2,3,4-tetrahydrophenanthrene‡.	F	1, 000	0/8
657	1-Keto-2-methoxymethylene-7-methoxy-1,2,3,4-tetrahydrophenanthrene.	F	1, 000	0/8
2116	2,7-Dinitrophenanthraquinone	E	1, 000	0/8
2118	2-Nitrophenanthraquinone	E	1, 000	0/8
	Heterocyclic compounds related to phenanthrene			
92x	α-Naphthoquinoline†	N	1, 000	1/8
920	β-Naphthoquinoline†	E	350	0/8
921	β-Naphthoquinaldine†	E	1, 000	0/8
1658	Phenanthridinium 1553†	H	80	1/8

*Expressed as: $\frac{\text{Number of mice dead}}{\text{Number of mice treated}}$.

†Soluble in water or acidulated water.

‡Soluble in dilute alkaline solution.

Discussion

None of these simpler analogs of colchicine, which represent degradation of its three-ring nucleus, appeared to have a high degree of potency in damaging Sarcoma 37 following a single injection. Of the biphenyls and phenanthrene derivatives, the only active compounds were those that had heterocyclic rings or some other functional group (guanidine or quinone) that apparently accounted for their activity. In view of our experience with many varieties of compounds with no relationship to the basic ring structure of colchicine, but containing similar functional groups, the ac-

tivity of these compounds seems to be more related to these groups than to the biphenyl or phenanthrene structure.

Haddow and Robinson (*5*) reported negative results with benzidine (NCI-3202) on spontaneous mammary tumors and Sarcoma 180. Boyland (*6*), however, reported inhibition of spontaneous tumors with this compound. He also reported 91 percent ($P=0.02$ using t test) inhibition of tumors with γ,γ-dipyridyl, a compound isomeric with α,α'-dipyridyl (NCI-335), the only compound related to biphenyl found by us to be potent in damaging Sarcoma 37. The findings with the phenanthrene derivatives supplement the reports, summarized by Dyer in her monograph (*7*), with this type of derivative.

The tropolone ring structure has a functional group (α,β-unsaturated ketone) which we have found to be associated with activity against Sarcoma 37 in other types of compounds. The number of simple tropolones examined is too small to permit conclusions regarding the importance of this ring structure itself in the activity of the colchicine molecule.

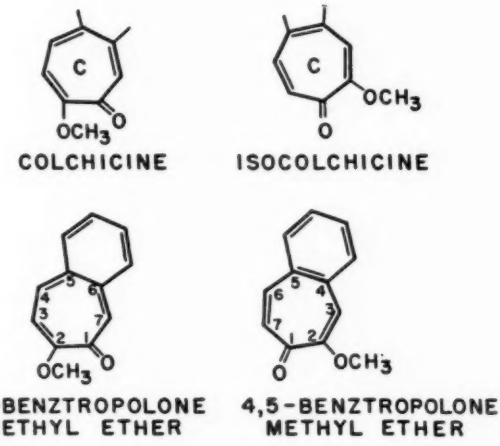
Of the six true tropolones reported here, only the *o*-bromo compound (NCI-3065) and γ-thujaplicin (*p*- or *5*-isopropyltropolone, NCI-2390) were active. None of the benztropolones (see table 3) produced damage. This would be in accord with the chemical properties of these two types of tropolones. Tarbell and Bill (*8*) have shown that the fused benzene ring stabilizes the resonance in the tropolone ring so that the benztropolone no longer reacts as do simple tropolones or colchicine.

In the case of the 4,5-benztropolone methyl ether (NCI–2430), the fused aromatic ring is in the same relative position as the B ring of isocolchicine rather than that of colchicine (text-fig. 3). Since the *iso* forms of the colchicine derivatives examined have all been inactive (*3*), it is conceivable that the 5,6-benztropolone methyl ether, more analogous to colchicine, might exhibit tumor-damaging potency. Unlike colchiceine, the two benztropolones discussed here (NCI-2469 and 2281) can form only one methyl ether.

NCI-3066, which represents a reduced tropolone ring, was also inactive. This is in accord with the greatly reduced activity of hexahydrocolchicine and hexahydrocolchiceine (*3*), in which the tropolone C ring of colchicine is reduced.

Lettré (*9*) has reported negative results against fibroblasts in tissue culture with tropolone and tropolone methyl ether. Katsura *et al.*, on the other hand, have recently published three papers (*10–12*) describing the effects on the Yoshida sarcoma of a considerably larger series of tropolones. They found *o*-bromotropolone (NCI-3065) to be negative against this ascites tumor. This is consistent with our finding that a dose greater than the MTD was required to damage Sarcoma 37. They did not report on γ-thujaplicin, but got essentially negative results with its two isomers, α-thujaplicin and hinokitiol (β-thujaplicin). Colchicine-like effects and/or increase in survival were reported with tropolone methyl ether, *p*-aminotropolone and its diacetate, and *o*-bromo-*p*-aminotropolone. Only the last compound was active at doses well below the lethal.

Except for the *o*-bromo compounds, all the active compounds reported by Katsura *et al.*, and by us, represent substitution in the *para* position of the tropolone. γ-Thujaplicin, in particular, has a branched chain in this position which could represent a remnant of the A and B rings of colchicine (see text-fig. 1). It might be of interest to determine the activity of some *meta*-substituted compounds of γ-thujaplicin. Since Katsura *et al.*, have reported a much greater activity and lower toxicity for an *o,p*-disubstituted compound than for either the *o*- or *p*- derivative itself, such disubstituted compounds might yield interesting results.



TEXT-FIGURE 3.

The thujaplicins are of additional interest. They are components of the wood of Western red cedar (*Thuja plicata*, Don) and have antibiotic and fungicidal properties that are considered to be of significance in the resistance of this wood to rot (*13–15*).

Summary

Sixty-nine compounds were examined for potency in damaging Sarcoma 37 following a single subcutaneous injection in CAF_1 mice bearing six-day-old tumor implants. They included 30 biphenyl (15 fluorene), 11 tropolone, and 28 phenanthrene derivatives and analogs. With the active compounds reported here, a dose near or above the maximum tolerated was required to produce damage.

α,α'-Dipyridyl, *9-aminofluorene*, and *9-methyl-9-aminofluorene* were the only compounds related to the biphenyls that were active. In the phenanthrene group, *1-methyl-5-(9-phenanthryl)-biguanide·HCl*, *phenanthraquinone*, *o-*, *m-*, and *p-phenanthroline* were active. *o-Bromotropolone*, *γ-thujaplicin* and *1,2-cycloheptanedione*, in the tropolone series, gave positive results.

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Spontaneous and Induced Tumors in Strain ST Mice[1, 2]

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Spontaneous lesions of the stomach of mice, except for adenomatous lesions of the glandular stomach found in strain I (*1*, *2*) and spontaneous gastric neoplasms reported by Strong (*3*) in strain BRS, are rare. Engelbreth-Holm [4] has reported lesions occurring in strain ST (Street) mice spontaneously and following treatment with carcinogenic agents. In mice of sublines that he used for various experiments with carcinogens, gastric ulcers with proliferation of the gastric epithelium were found in the stomachs of some of the animals, and in a few animals adenocarcinomas of the stomach were noted. (No exact incidence data were supplied.) Engelbreth-Holm and Poulsen (*4*) found "hyperplasia of the stratified squamous epithelium of the fundus of the stomach with keratosis, a tendency to papilloma formation" in almost all the mice fed a 1 percent solution of 9,10-dimethyl-1,2-benzanthracene. The present experiment was prompted by the thought that the appearance of gastric lesions might be hastened by the intravenous injection of a carcinogenic hydrocarbon. At the same time the effect of the intravenous injection of the hydrocarbon on the production of pulmonary tumors could be studied. The results obtained when a group of strain ST mice were injected with 20-methylcholanthrene are presented here. The findings in a group of untreated mice and in a group of breeding animals are included for the purpose of comparison.

Materials and Methods

The strain ST mice used in this experiment were produced in the colony established in the genetics laboratories of the National Cancer Institute from a breeding litter obtained from the Radium Hospital of Copenhagen through the courtesy of Dr. Engelbreth-Holm in August 1947. These mice originated from a group of mice bred in Denmark by a Mrs. Street. Animals received from Copenhagen were of the twenty-third brother ×

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[2] This manuscript was prepared for publication while two of the authors (M. K. D. and M. K. B.) were Summer Investigators at the Roscoe B. Jackson Memorial Laboratory in Bar Harbor, Maine.

[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[4] Personal communication.

sister inbred generation, and those used in the present experiment were of generations F_{24} through F_{27}.

Three groups of strain ST mice were injected intravenously with 20-methylcholanthrene. The first group, containing 40 males and 51 females, was injected at 10 weeks of age with 2 mg. of 20-methylcholanthrene dispersed in 0.2 cc. of distilled water containing 0.1 percent methocel and 0.01 percent aerosol; the second group, containing 28 males and 30 females, was injected at 8 weeks of age with 0.5 mg. in 0.5 cc. of horse serum; and the third group, containing 25 males and 26 females, was injected at 8 weeks of age with 0.2 mg. in 0.2 cc. of horse serum.[5] The untreated group consisted of animals set aside at weaning to be observed for the development of spontaneous lesions. Records are available for 91 animals (58 males and 33 females) in this group. The breeding group consisted of 103 animals (25 males and 78 females). The animals were housed in wooden cages, 8 animals to a cage, and were continuously supplied with tap water. The injected and breeding animals were fed Derwood food pellets, and the untreated animals were fed Purina laboratory chow.

It was planned that autopsies of the injected animals would be performed at a uniform age of one year, but the development of severe dermatitis in some of the mice necessitated their autopsy at an earlier date. At autopsy, all organs were examined but particular attention was given to the stomachs, which were opened and were examined for the presence of gastric lesions; to the lungs, which were examined for the presence of pulmonary tumors; and to the subcutaneous tissues for the presence of tumors there. Microscopic sections were made of all subcutaneous tumors, and of at least one pulmonary tumor from each individual in which pulmonary tumors were found. Sections were made of stomachs only when some alteration of the mucosa of the stomach was observed grossly, and sections of lymph nodes, liver, or spleen were made when these organs were enlarged. The untreated virgin mice were autopsied when they were moribund or were found dead. The breeding animals were autopsied when tumors, dermatitis, or other types of lesions developed. Tissues taken at autopsy were fixed in Fekete's modification of Tellyesniczky's fixative (70 percent ethyl alcohol, 20 parts; formalin, 2 parts; glacial acetic acid, 1 part) or 10 percent formalin, and stained with hematoxylin and eosin.

Results and Discussion

The types of tumors found in the injected strain ST mice and in the untreated virgin and breeding mice of this strain are listed in table 1. Forty animals (15 males and 25 females) injected with 2 mg. of 20-methylcholanthrene, 4 animals (3 males and 1 female) injected with 0.5 mg., and 5 animals (3 males and 2 females) injected with 0.2 mg. were not included

[5] The dispersions of 20-methylcholanthrene used in this experiment were prepared by Dr. Egon Lorenz of the National Cancer Institute.

TABLE 1.—*Types of tumors found in treated and untreated strain ST mice*

Sex	Type of treatment	Number of mice	Average age at death (*months*)	Type of lesion	Mice with tumors		
					Number	Percent	Average age at death (*months*)
♂	Injected intravenously with 20-methylcholanthrene (2 mg.)	25	9.7	Pulmonary tumors	17	68.0	10.8
♀	Injected intravenously with 20-methylcholanthrene (2 mg.)	26	9.7	Pulmonary tumors Mammary gland tumors Leukemia	21 2 3	80.7 7.7 11.5	10.4 10.5 8.0
♂	Injected intravenously with 20-methylcholanthrene (0.5 mg.)	25	8.4	Pulmonary tumors Leukemia	13 1	52.0 4.0	11.1 12.0
♀	Injected intravenously with 20-methylcholanthrene (0.5 mg.)	29	10.4	Pulmonary tumors Leukemia	22 1	75.8 3.4	11.9 5.0
♂	Injected intravenously with 20-methylcholanthrene (0.2 mg.)	22	7.0	Pulmonary tumors	2	9.0	7.5
♀	Injected intravenously with 20-methylcholanthrene (0.2 mg.)	24	9.5	Pulmonary tumors	8	33.3	12.0
♂	Untreated virgins	58	9.4	Pulmonary tumors	1	1.7	21.0
♀	Untreated virgins	33	12.1	Pulmonary tumors Mammary gland tumors Leukemia	1 2 2	3.0 6.0 6.0	18.0 13.5 13.5
♂	Untreated breeders	25	9.8	Pulmonary tumors Leukemia	3 1	12.0 4.0	10.0 11.0
♀	Untreated breeders	78	8.9	Pulmonary tumors Mammary gland tumors Leukemia	3 26 1	3.8 33.3 1.2	9.0 9.9 10.0

because of death immediately following injection or because of failure to obtain autopsies owing to extreme autolysis of the animals when found dead.

The intravenous injection of 20-methylcholanthrene significantly increased the incidence of pulmonary tumors in the strain ST mice in the present experiment. Seventeen of the 25 males and 21 of the 26 females injected with 2 mg. of 20-methylcholanthrene and autopsied at average ages of 10.8 and 10.4 months had pulmonary tumors, while 13 of the 25 males and 22 of the 29 females receiving 0.5 mg. and 2 of the 22 males and 8 of the 24 females that received 0.2 mg. and were autopsied at average ages of 11.1, 11.9, 7.5, and 12 months, respectively, had pulmonary tumors. These results were compared with those obtained for untreated animals where 2 (1 male and 1 female) of 91 virgin animals had pulmonary tumors when examined at ages of 21 and 18 months, and 6 (3 males and 3 females) of 103 breeding animals had pulmonary tumors when examined at average ages of 10 and 9 months. The incidence in the untreated animals was comparable to the 3 percent found by Engelbreth-Holm and Rask-Nielsen (*5*). The increase in the incidence of pulmonary tumors following intravenous injection of 20-methylcholanthrene was also reflected in the number of tumor nodules found per individual, as shown in table 2.

TABLE 2.—*The occurrence of multiple pulmonary tumors in strain ST mice after injection of 20-methylcholanthrene as compared with the occurrence of such tumors in untreated animals*

Group	Mice with given number of pulmonary tumors											
	0	1	2	3	4	5	6–15	16–25	26–35	36–45	46–55	55–60
2 mg.	13	3	4	6	3	1	8	6	2	3	1	1
0.5 mg.	19	8	2	5	4	5	9	2	....	....	....	....
0.2 mg.	36	6	2	2	....	....	....	....	....	....	....	....
Untreated	186	8	....	....	....	....	....	....	....	....	....	....

Leukemia was observed grossly in small numbers of both the injected and the untreated animals. All the cases observed were histologically similar to those described by Rask-Nielsen and Gormsen (*6*) in strain ST mice and classified as typical stem-cell leukemias.[6] The incidence in the two groups did not differ significantly and was in close agreement with the incidence of 1 to 2 percent found by Engelbreth-Holm and Rask-Nielsen (*5*). The latter investigator (*7*) also did not obtain an increase in the incidence of leukemia in strain ST mice following injection of 20-methylcholanthrene into the flank or into the mammary gland region.

The intravenous injection of 20-methylcholanthrene had no effect on the incidence of mammary gland tumors. This finding was in agreement

[6] The authors are indebted to Dr. Thelma B. Dunn, of the National Cancer Institute, who made the diagnoses of leukemia in these animals.

with the results obtained by Rask-Nielsen (*7*, *8*). In the present experiments, two of the 26 strain ST female mice injected with 2 mg. of 20-methylcholanthrene developed mammary gland tumors at 10 and 11 months. None of the females injected with either 0.5 mg. or 0.2 mg. of 20-methylcholanthrene developed mammary gland tumors. Among the untreated animals, two females in the group of 33 virgins developed mammary gland tumors at 12 and 14 months, and 26 of the 78 breeding females developed mammary gland tumors at an average age of 9.9 months. The number of animals developing mammary gland tumors does not differ significantly from the number reported by Engelbreth-Holm and Rask-Nielsen (*5*). They have reported incidences of 7 percent for mammary carcinomas in nonbreeding animals and of 35 percent in breeding animals. The increased incidence of mammary gland tumors observed among breeding females when compared with virgin females was similar to that previously reported for strain HR (*9*) but somewhat less than that reported for strain A (*10*). It was somewhat comparable with that reported in strain C3Hf (formerly C3Hb) without the milk agent (*11*). The incidence of mammary gland tumors in strain C3Hf females was 2 percent in virgin females and 38 percent in breeding females.

Lesions have been observed grossly in the stomachs of 3 (1 male and 2 females) of the 51 animals receiving 2 mg. of 20-methylcholanthrene, in 4 (females) of the 54 animals receiving 0.5 mg., and in 2 (females) of the 46 animals receiving 0.2 mg. Seven (6 males and 1 female) of the 91 untreated virgin animals and 3 (females) of the 103 untreated breeding animals showed similar lesions. Histologic sections of these lesions have been described briefly as follows: "None of the sections of the stomach showed evidence of carcinoma. Nearly all, however, showed some degree of gastritis of the glandular chamber. In some this was characterized by superficial, minimal, focal erosions of the mucosa associated with an infiltration of inflammatory cells about the denuded areas. Other lesions showed a more pronounced inflammatory cell infiltration about areas of thickening, reduplication, atrophy, and ulceration of the mucosa. Areas of fibrosis of the mucosa may have been the end result of healing. Some of the glands were dilated and lined by cells which were hyperchromatic, as is characteristic of the lesion of chronic gastritis. In a number of specimens there was extension of the gastric glands into the submucosa. Amyloid was observed in the lamina propria in one specimen. The forestomach frequently showed hyperkeratosis, acanthosis, and papilloma." [7]

Summary

The following incidences of spontaneous tumors in untreated strain ST mice were observed in the present experiment. Incidences for pulmonary tumors were 3 percent in virgin females, 3.8 percent in breeding females, 1.7 percent in virgin males, 12 percent in breeding males; because of the small numbers involved these differences were not considered significant.

[7] The authors are grateful to Dr. Harold L. Stewart and Dr. Samuel M. Schlyen, of the National Cancer Institute, who have examined the histologic sections and described the observed lesions.

Those for leukemia were 4 percent in breeding males, 6 percent in virgin females, and 1.2 percent in breeding females. Incidences for mammary gland tumors were 6 percent in virgin females and 33.3 percent in breeding females.

The intravenous administration of 2 mg. of 20-methylcholanthrene increased the incidence of pulmonary tumors to 68 percent in males and to 80.7 percent in females. Lesser effects were observed with doses of 0.5 mg. and 0.2 mg.

The intravenous administration of 20-methylcholanthrene in the three different doses did not increase the incidence of leukemia, of mammary gland tumors, or of gastric lesions.

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Induced Adaptation in a Tumor: Specificity of the Change[1,2]

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It is generally accepted that the transplantation of tissues, either normal or neoplastic, depends in a major way upon the genetic constitution of both the tissue and the recipient. This principle, which was foreshadowed by Loeb and firmly established by the work of Little, Tyzzer, Strong, Bittner, Andervont, and others, has been ably reviewed and discussed by Little (*1*). It is presumed that "foreign" tissues contain "antigens" not present in the recipient and that such tissues arouse a defensive mechanism in the host that brings about destruction of the grafted tissue. It was presumed that transplantable tumors were relatively stable in this respect, except when spontaneous mutations occurred, until recent experiments by the authors indicated that the host environment may change the tumor in some unexplained manner, so that a change in the transplantability of the tumor results.

The authors observed that a tumor which otherwise behaved in accordance with the usual genetic restrictions on transplantation, underwent a change in transplantability during growth outside the strain of origin of the tumor (*2*). The change, which was seen as an enhancement of the tumor's inherent ability to grow in a limited number of resistant backcross mice, could be produced at will by growing the tumor in an F_1 hybrid mouse. Further investigation disclosed that the change represented a permanent alteration in the properties of the tumor tissue (*3*). The enhanced transplantability persisted through many generations and was not reversed by growth in the parent inbred strain. New experiments have been performed to ascertain if the phenomenon is of a specific nature. The results of such experiments are reported herein.

Materials and Methods

The technical details have been given previously (*2*, *3*) and will be omitted here except for the major features.

The tumor used was a mammary adenocarcinoma called C3HBA, which has been previously described in detail (*4*, *5*). It originated in strain

[1] Received for publication April 14, 1953.

[2] Part of this data was presented before the Annual Meeting of the American Association for Cancer Research at Chicago, Ill., April 9-11, 1953.

[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

C3H/An (*6*) and, upon transplantation, grew rapidly in all strain C3H mice and in all the F_1 hybrids here used. It has never grown in strains BALB/c or DBA/2 (nor in 3 other strains) although it has been inoculated into such mice many times. During the 7 years that various aspects of the growth of this tumor have been under investigation, the stock tumor has been continuously maintained by serial transplantation in strain C3H. Frequent tests (one of which was made at the conclusion of this work) have thus far not revealed any morphologic change in the stock tumor or any spontaneous change in its transplantability.

The animals used included inbred strains C3H/He, BALB/cN, and DBA/2Lw (*6*), hereinafter called strains C3H, C, and D, respectively.[4] Two kinds of F_1 hybrids were obtained by mating strain C3H females with either strain C or strain D males—thus obtaining animals designated (C3H × C)F_1 or (C3H × D)F_1 respectively. Two kinds of resistant backcross mice were obtained by mating females of one or the other of the F_1 hybrids with males of the corresponding resistant strain (*i.e.*, the strain foreign to the tumor), thus obtaining animals designated (C3H C × C)BC or (C3H D × D)BC respectively.

The tumor tissue was subjected to 3 different conditions: 1) maintenance in strain C3H (stock tumor); 2) growth in (C3H × C)F_1 hybrids (adapted tumor); or 3) growth in (C3H × D)F_1 hybrids (adapted tumor). Adaptation was induced separately for each separate experiment by inoculating a small group of F_1 hybrids (young adults of either sex) and allowing the tumor to grow for 2 to 3 weeks; the F_1 hybrids then were used as donors of tumor tissue which was inoculated into backcross recipients. No more than 1 generation of growth in the F_1 hybrids occurred in any experiment. The tumor grafts were all small, uniform doses of brei inoculated subcutaneously.

Transplantability of the tumor was always evaluated in backcross recipients because among such animals only a limited number are susceptible, whereas all the F_1 hybrids are susceptible [see (*1*) or (*9*) for the principles involved]. Such recipients were inoculated when 8 to 12 weeks old and examined at frequent intervals thereafter for the presence, or absence, of a progressively growing tumor. Observations were continued until the last tumor-bearing animal in a group had died, whereupon the remaining tumor-free animals were killed.

An additional aspect of the genetic constitution of the recipient animals was introduced by the fact that the segregation of specific genes in the backcross populations led to variations in color. Half of the (C3H C × C)BC mice would be expected to be albino, and the rest to be colored,

[4] The two foreign strains selected for this work seem to represent a good choice from the standpoint of interstrain relationships because they have approximately the same relationship to strain C3H (*7*). However, a complication was introduced by this selection. Strain D carries the mammary tumor agent whereas strain C does not. Although the tumor itself contains the agent (*4*), its transplantability and growth rate are influenced by the presence or absence of the agent in the host (*2, 8*). It did not seem likely that this difference in the male parents would cause a discernible difference in this work, because all the animals had a strain C3H mother or grandmother, and therefore carried the agent. Nevertheless, as a check on this possibility, we used strains DBA/2Lw (with the agent) and DBA*t*/2Lw (without the agent) in the production of the hybrids. The classes of animals thus distinguished were equally distributed through the experiments. No difference in the data ascribable to this difference in paternal parents was seen, and such classes of animals are aggregated in the data given.

equally distributed between black agouti and brown agouti. It should be noted that not all the segregants can be identified in this population. Among the white animals, half carry the gene for black and half carry the gene for brown but action of these genes does not become manifest since the animals are homozygous for albinism. Thus in the (C3H C × C)BC recipients, the effect of the gene for color can be seen in all the animals but the effect of black or brown can be seen in only half. Among the (C3H D × D)BC animals 8 coat colors based upon segregation of the 3 genes involved would be expected. These colors were approximately equally distributed among black, black agouti, dilute black, dilute black agouti, brown, brown agouti, dilute brown, and dilute brown agouti. This latter population offered an especially good opportunity to extend the observations on specificity beyond the range of interstrain differences. Accordingly, as the observations in this group accumulated, recipients were deliberately distributed so as to have the same number of males and females of each color and the same number of each color in the various experimental categories. In the (C3H C × C)BC group the numbers of animals in each class are approximately balanced although no deliberate balance was introduced. In both populations of recipients, individual records of the animals were kept so that the exact distribution of positive cases among the various color segregants is known.

Because of limitations of animal space and the difficulties of obtaining these animals in large numbers, it was necessary to perform the experiments with relatively small groups of animals and to aggregate the results of corresponding experiments. Each of the 6 groups of recipients shown in table 1 represents 3 or more experiments. Specifically, the 3 groups in the middle column represent 16, 17, and 3 separate experiments, respectively, and those in the right column represent 13, 17, and 35 experiments, respectively. Individual experiments varied from the general pattern only in minor details and the earliest experiments were in keeping with the final ones.

Interstrain Aspects of Specificity

The data comprise observations on more than 2,300 recipients and, as will become apparent, several factors have influenced the results. Some of the factors are of an intrastrain character and some are interstrain. Confusion is lessened if the animals are grouped in different ways for varied purposes and attention is focused on one aspect at a time. This method has been adopted for the presentation which follows. Table 1 shows the first and simplest arrangement of the data.

Because an unusual number of statistical tests were required in evaluating the data, the text has been simplified by omitting all but the most important statistical considerations. More than 60 statistical tests for significance were applied to the data.[5] These tests gave results that were

[5] The authors are greatly indebted to Mr. Marvin Schneiderman of the Biometrics Section, National Cancer Institute, who assisted with all the statistical analyses and who performed the more difficult computations.

TABLE 1.—*Backcross recipients succumbing to a progressively growing adenocarcinoma C3HBA after transplantation from one of three types of donors**

Donor of tumor graft	Backcross recipients					
	(C3H C × C)BC			(C3H D × D)BC		
	Number of		Percent positive†	Number of		Percent positive†
	Tumors	Hosts		Tumors	Hosts	
C3H (stock tumor)	30	302	10	108	384	28
(C3H × C)F_1	69	258	*27*	137	384	36
(C3H × D)F_1	45	218	21	348	800	*44*

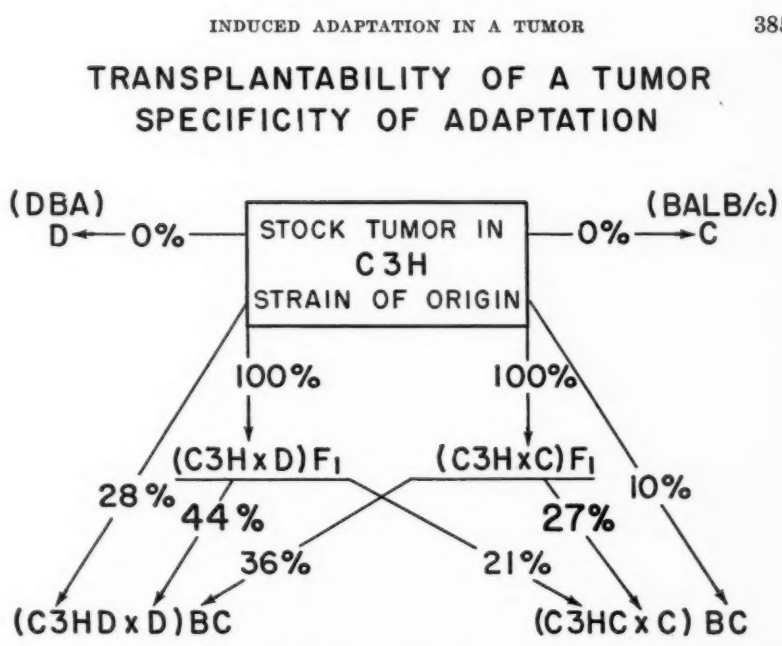
*The donors were either the strain of origin of the tumor or one of two F_1 hybrids derived from that strain and a foreign strain. The recipients were either of two resistant backcrosses corresponding to the F_1 donors.

†Italicized figures indicate that the highest result in each recipient was obtained when the donor corresponded to the recipient with respect to foreign strain. The differences are significant.

all in accordance with statements made in the text, but only the more critical ones will be mentioned specifically.

Results and Discussions

Text-figure 1 affords the easiest visualization of the relationships among the various experiments and their results. The two 0 percent results and the two 100 percent results are what would be expected from genetic principles, and they have been verified many times with these materials. The critical data are the numbers of animals succumbing to the tumor among two populations of backcross recipients, one derived from strain C and one derived from strain D. The critical variations in the data are those indicated by the 6 results shown in the lower part of the diagram. These 6 results consist of 2 sets of 3, one set for each type of backcross recipient. It is apparent that in each set the recipients are the same but the donors differ, and that for each recipient there is one F_1 hybrid donor that corresponds with respect to the foreign strain involved and one that is noncorresponding. That there are differences *between* the sets would have been expected from well-known genetic principles, but the differences *within* the sets would not have been expected. The important differences are all statistically significant except one—the difference between 21 percent and 27 percent in (C3H C × C)BC recipients ($P=0.127$). In that case, the statistical limitations are such that a numerical value between 10 percent and 27 percent, which was significantly different from both, could not have been obtained without using larger numbers of animals—a requirement that could not be met with the facilities available at the time. However, the difference obtained appears to be trustworthy because of other considerations: the same trends were shown by the individual experiments from which these results were accumulated, and the same trends were shown by the results in the other backcross. Statistical analysis indicates that this latter correspondence is significant ($P=0.01$).



TEXT-FIGURE 1.—The results of transplantation of the tumor from various donors to various recipients. The arrows indicate the donor-to-recipient relation, and the figures on the arrows indicate the percentage of recipients succumbing to the tumor. Note that the highest result attained in one backcross population was 44 percent and the highest in the other was 27 percent.

The diagram is based upon the data shown in table 1, where the results are arranged in their simplest form to emphasize the differences that result from using different foreign strains (*i.e.*, foreign to the tumor) in producing the animals. In this arrangement attention is directed to the fact that the backcross recipients were inoculated with tumor tissue furnished by one of 3 classes of donors: 1) strain C3H, in which stock tumor was maintained; 2) (C3H × C)F_1 hybrids, in which the tumor had been adapted by one generation of growth; or 3) (C3H × D)F_1 hybrids, in which the tumor was likewise adapted. In other words, stock tumor was either passed directly into the 2 classes of recipients or it was passed indirectly through one of the F_1 hybrids and thence into the backcross recipients (see text-figure 1).

As was expected, the tumor grew in different proportions of the 2 classes of recipients. This difference, though significant, does not represent a new principle. It serves to reaffirm the generally accepted concept (*1*) that these proportions represent relative differences between the tumor tissue and the *particular* host to which it was transplanted (differences that vary from strain to strain), and not generalized differences between the tumor and all hosts. Thus there is a characteristic behavior of the unchanged tumor that is different in different populations of hosts, and transplantability of the adapted tumors into either class of backcross recipients

should be compared with the transplantability of stock tumor into the same kind of recipient.

Stock tumor grew progressively in 10 percent of (C3H C × C)BC recipients, whereas after adaptation in a (C3H × C)F_1 hybrid the tumor grew in 27 percent of such recipients. Neither of these results is significantly different from results previously obtained under similar circumstances (*2*). In contrast, when the tumor had been adapted in a (C3H × D)F_1 hybrid it grew in only 21 percent of (C3H C × C)BC recipients. (As remarked earlier, there is a statistical weakness here but, on grounds which appear to be sufficient, the authors believe that the difference is real.) Thus the adaptive enhancement of transplantability was seen when either F_1 hybrid was the donor, but the degree of enhancement was greater when the donor and recipient corresponded with respect to the foreign strain from which they were derived.

In the (C3H D × D)BC recipients similar relationships between the results were observed but at different numerical levels, and in this case all the differences are statistically significant. It was again observed that the degree of enhancement was greater (44 percent compared with 36 percent) when the adapted tumor was transferred from the corresponding donor than in the alternate case. Statistical analysis indicates that this correspondence is significant ($P=0.01$).

From the results presented thus far the following conclusions seem justified: 1) The phenomenon of adaptation as originally observed has been confirmed.[6] 2) The phenomenon is not limited to the specific hybrids originally used but may also be seen when using a different F_1 hybrid donor, a different backcross recipient, or both. 3) The donor and recipient need not be derived from the same foreign strain. In the latter sense the phenomenon is not specific, but in the light of further results (presented below) it appears better to look upon this as a type of cross reaction rather than as nonspecificity. 4) Although enhancement occurred when the donor and recipient did not correspond, a greater degree of enhancement was observed when they did. In this quantitative sense, the phenomenon can be said to be specific with respect to the genetic constitution of the host. Extrapolation beyond the limits of this work is speculative but, if the phenomena observed are of an immunologic kind, one would expect that specificity might be a matter of degree because it is accepted that immunologic specificity may be manifested only by strength of reaction. In the following section of this paper it will be shown that there also appears to be a qualitative difference between the corresponding and noncorresponding systems.

Intrastrain Aspects of Specificity

In the previous section the animals were grouped, on the basis of genetic constitution, into large populations and attention was directed to those characteristics common to the individuals comprising the populations.

[6] The phenomenon has also been confirmed in another laboratory and with 2 different tumors. (Personal communication and unpublished data of Dr. T. S. Hauschka.)

Balanced proportions of several different classes of individuals were included in each population of backcross recipients. The different classes, which can be identified by their color, arise because of the well-known segregation of genes that occurs in the backcross generation. Attention to this feature permits more detailed observations regarding the specificity of the stock tumor and the changes occurring during adaptation. In the following discussion this aspect is emphasized. The animals involved are the same as those in the preceding section, but they are grouped in a different way.

Results and Discussion

In order to clarify certain special features of the data with respect to specific genes, or linkage groups of genes, the animals were grouped in two different ways. In the first arrangement each of the several color classes was treated separately; thus each group differed from all other groups by one or more genes and the aggregate effects of the 2 or 3 genes involved could be seen. In the other arrangement the animals were grouped according to the presence of a single dominant gene or its recessive allele; thus each group differed from the corresponding group by only one known gene; the other differences were randomly distributed through the groups and therefore without effect. These two arrangements are presented separately in the following discussion.

Table 2 shows the results in the several color classes of recipients. It is noteworthy that the general statements of the previous section also apply, with some exceptions, to this more specific grouping of the data. Adapted tumors showed enhancement of transplantability in every color class of recipients. In general, enhancement followed the use of either F_1 donor but was greater with the corresponding donor. However, in 4 classes of the (C3H D $\times$ D)BC recipients there were exceptions to these generalizations. The 4 exceptions appeared as variations in the results of transferring tumor from a (C3H $\times$ C)F_1 (*i.e.*, noncorresponding) donor to the dilute black, dilute black agouti, brown, and brown agouti recipients. In the dilute black agouti, the results with tumor from the noncorresponding donor were higher than those with the corresponding donor; in the brown agouti recipients, the tumor from the noncorresponding donor was not different from stock tumor in transplantability; and in both the brown and dilute black recipients, the tumor from a noncorresponding donor yielded lower results than either stock tumor or correspondingly adapted tumor. These are very interesting variations but they are not reliable statistically. Further experiments bearing on this point are in progress.

The observation that each segregant yielded a quantitatively different set of results is similar to the observations on interstrain differences (except that in this case it extended to differences within the backcross population) and could be explained in the same manner. When stock tumor was used, the over-all average result was statistically compatible with an "expected genetic ratio" (*1*) for a certain number of gene pairs or

TABLE 2.—*Backcross segregants succumbing to adenocarcinoma C3HBA under varied conditions**†

Donor of the tumor graft	(C3H C × C)BC			(C3H D × D)BC							
	Color			Color							
	Albino	Brown agouti	Black agouti	Black	Black agouti	Dilute black	Dilute black agouti	Brown	Brown agouti	Dilute brown	Dilute brown agouti
C3H (stock tumor)	19/154 6%	5/73 11%	12/75 16%	5/48 10%	16/48 33%	19/48 40%	13/48 27%	17/48 35%	17/48 35%	7/48 15%	14/48 29%
(C3H × C)F_1	29/127 23%	21/67 31%	19/64 30%	16/48 33%	21/48 44%	18/48 38%	24/48 50%	12/48 25%	17/48 35%	11/48 23%	18/48 38%
(C3H × D)F_1	17/95 18%	14/61 23%	14/62 23%	38/100 38%	49/100 49%	48/100 48%	46/100 46%	39/100 39%	50/100 50%	37/100 37%	41/100 41%

*The data and conditions are the same as those in table 1, but are regrouped to show the results in the different color segregants.
†The results are given both as a percentage and as a fraction (the numerator equaling the number of tumors, and the denominator the number of animals inoculated).

factors (3 or 4 in the backcross to strain C and 2 in the backcross to strain D), but among the color segregants the results varied widely about such an over-all average. Theoretically it makes a great deal of difference whether these variations are real or accidental. In other words, one wishes to know whether the variations of results within a backcross population could have been accidentally derived from a statistically homogeneous population of numbers.

Postponing attention to particular genes and considering the results merely as populations of variable numbers, appropriate statistical tests were applied to determine whether the variations in results *within* each group of recipients were random or significant. In the (C3H C × C)BC recipients the statistical significance was weak; the variations in results following transplantation of stock tumor were greatest but there is a 7.5 percent probability that they were due to chance. Corresponding probabilities for the two other groups were about 40 and 60 percent. It can be said only that the appearance of smaller variations after adaptation is in line with other observations. On the other hand, there were significant variations in the results in (C3H D × D)BC recipients. The variations that were seen after transplanting stock tumor were significant at the 1 percent level. One may therefore conclude that among these segregants there is an *unequal* distribution of the factors controlling the transplantability of the *stock* tumor. This situation appears to have been changed by adaptation in the corresponding donor. Analysis of the results following transfer of tumor from a (C3H × D)F_1 donor to the (C3H D × D)BC recipients indicates a 50 percent chance that the variations were random. This suggests that among these segregants there is an *equal* distribution of the factors that control the transplantability of the *correspondingly adapted* tumor. The change must have occurred in the tumor tissue and it appears that, in some obscure way, it led to a more uniform reaction between the tumor and the various segregants in this backcross population. The results following the transplantation of tumor adapted in the noncorresponding donor were intermediate statistically. Analysis indicated a probability of 0.08 that the variations in that population were random. The tentative conclusion of the authors is that the *noncorresponding adaptation* did not produce a uniform reaction between the tumor and the various segregants, at least not to the degree that followed corresponding adaptation. This tentative conclusion was supported by other aspects of the data to be given later.

The data, when seen from this viewpoint, suggest the conclusion that the phenomenon of adaptation exhibits a degree of specificity which extends to the segregants within a backcross population and which, although generally expressed quantitatively, may have a qualitative aspect.[7] Variations in the results among the segregants is greatest following transplantation of stock tumor; this appears to represent a specificity innate in the tumor. The shift in results following adaptation differs

[7] Here and subsequently, the term "qualitative" is used in its logical sense, *e.g.* enhancement may be present in one situation and absent in another, or one population may be homogeneous whereas another is nonhomogeneous.

among the segregants; this appears to be a specificity that is expressed by quantitative variations in the adaptive process. This latter set of differences leads to a greater uniformity in the results among the recipients and seems to be a partial loss, or alteration, of the innate specificity of the tumor tissue. The observation that the shift toward uniformity in the results was less when the noncorresponding donor was used suggests a qualitative aspect in adaptation. The same impression was gained when the data were arranged in another manner.

The final manner in which the data were arranged is based upon the fact that, although more than one gene is concerned in the segregation in the backcross generation, it is possible to group all the recipients of one kind into two groups so that, for any one of the genes, one group carries the dominant allele and the other carries the corresponding recessive allele. This is the method commonly used in determining "linkage," or the tendency for certain characters to be associated genetically. As one example, all the (C3H D × D(BC recipients can be divided into two groups, one of which carries the gene for black and one of which carries the gene for brown. In that case the groups differ by only those characters associated with the linkage group of genes where "black" is located. Characters that are associated with "dilute" and "agouti" are presumably equally distributed between the groups and do not affect the comparison. It should be noted that, in this arrangement of the data, the same animals are regrouped for each successive gene and an apparent increase in the total number of animals appears in the table. This sort of dichotomy was used in the construction of table 3 in order to bring out more clearly certain aspects of the data which were obscure in the other arrangements

In the (C3H C × C)BC recipients, this arrangement reveals only two points that are new, although the general statements made in previous sections of this paper apply for each color. Only one comparison between colors showed a statistically significant difference; that was the case when the data for all 3 donors were aggregated. When all were added, the slight superiority of colored over albino that was present in the case of each donor produced a significant difference between the totals. The other point worthy of note is that there was no difference between black and brown in these animals. This became interesting when it was seen that there was an important difference between black and brown in the (C3H D × D)BC animals.

In the (C3H D × D)BC recipients some interesting differences were seen when the data were arranged in this fashion. No linkage or association with dilution was detected and in this case only the generalizations previously stated applied. Bittner (*10*) and Little and Strong (*11*) detected evidences of linkage with dilution in their experiments; however, that is not a contradiction because they worked with different materials. The point emphasized by the contrast between their work and ours is that. as stated earlier, the factors controlling the results of such experiments are specific for the particular combination of host and tumor employed. On the other hand, the data of table 3 indicate linkage with both black and

TABLE 3.—*Backcross recipients succumbing to adenocarcinoma C3HBA under varied conditions, arranged to show an apparent qualitative difference between the corresponding and the noncorresponding adaptions* * † ‡

DONOR OF THE TUMOR GRAFT	$(C_3H \times C) BC$ SEGREGANTS				$(C_3H \times D) BC$ SEGREGANTS					
	Colored	Albino	Black	Brown	Dilute	Non-dilute	Black	Brown	Agouti	Non-Agouti
C_3H (Stock tumor)	14 % 20/148	6 % 10/154	16 % 12/75	11 % 8/73	28 % 53/192	29 % 55/192	28 % 53/192	29 % 55/192	31 % 60/192	25 % 48/192
$(C_3H \times C)F_1$	31 % 40/131	23 % 29/127	30 % 19/64	31 % 21/67	37 % 71/192	34 % 66/192	41 % 79/192	30 % 58/192	42 % 80/192	30 % 57/192
$(C_3H \times D)F_1$	23 % 28/123	18 % 17/95	23 % 14/62	23 % 14/61	43 % 172/400	44 % 176/400	45 % 181/400	42 % 167/400	47 % 186/400	41 % 162/400

*The data and conditions are the same as those in tables 1 and 2 but are regrouped to bring out a different aspect. Animals carrying a specific gene may be contrasted with animals carrying the corresponding allele. Note that because more than one segregating gene is involved, a single animal enters into more than one comparison (see text).

†The results are given both as a percentage and as a fraction (the numerator equaling the number of tumors, and the denominator the number of animals inoculated).

‡In the 2 columns at the right (and only there) the arrows connect pairs of results that are *not* significantly different; other pairs in those columns are significantly different.

agouti but *only* in the case of tumor transferred from the noncorresponding donor. In the black-brown and agouti-nonagouti columns of the (C3H D × D)BC part of the table (and only there) the arrows connect pairs of numbers that are not significantly different; other pairs (in those columns) are significantly different. It is apparent that when the corresponding donor was used enhancement was seen in all recipients, but when the noncorresponding donor was used enhancement was not seen in the brown or nonagouti animals. This same effect was seen in a different way when the animals were divided into 3 groups: 1) those carrying either black or agouti but not both, in which the generalized or distributed effect was seen; 2) those carrying both black and agouti, in which significant enhancement followed the use of either hybrid donor; and 3) those carrying neither black nor agouti, in which significant enhancement followed the use of only the corresponding hybrid donor. (The details of this last grouping can be extracted from table 2.)

Consideration of this last aspect of the data suggests that the specificities studied may have a qualitative as well as quantitative character. However, many obscurities remain and this last point can be accepted only tentatively and with caution.

General Discussion

Although these experiments revealed some new aspects of the adaptive phenomena under investigation, they did not furnish a basis for final

interpretation of the underlying nature of the change. The possibility that the underlying mechanism is an environmental selection of tumor cells having certain potencies from among a spontaneously variable population of cells remains an open question. At present it appears that this question will probably remain moot for some time, but as data accumulate a new aspect arises for consideration. This concerns the mathematical consequences of applying such an interpretation where a large number of factors are involved.

A decision that the phenomena were adequately explained by a selective mechanism would require the presumption that every, or nearly every, small randomly-selected dose of stock tumor contains a sufficient number of all variants to account for the manifold results. Such a premise can be accepted easily as long as the number of factors involved remains relatively small. However, as the number of elements involved in the various combinations is multiplied the resulting possibilities become very numerous. There is reason to believe that if new strains of mice were introduced into the experiments the total number of factors required to satisfy all variations would again be increased. It is apparent that if such a line of reasoning were extended the mathematical requirements would soon get out of hand. Such considerations suggest that the selective mechanism may not be as likely an interpretation as it at first appeared.

The questions that arise from the use of the word "mutation" in relation to the phenomena reported also remain unanswered. These questions cannot be answered until the possibility of selection is ruled out and this has not been done. The fact that these results permit discussion in terms of genes, or genic complexes, lends an air of genetic exactness that may seem to bring the work closer to interpretation on the basis of induced mutation in the cells of the tumor, but that is largely illusory. It can no longer be doubted, if judgment is based upon the usual criteria, that there are differences between the genetic constitution of the stock tumor and that of the adapted tumors. Furthermore, some of the differences appear to be linked, or associated, with known genes for coat color. However, this appearance of relatively exact genetic differences does not require that the differences in the individual cells, as contrasted with whole tumors, arose during the adaptive process. Variant cells may have existed in small numbers in the stock tumors. This would not alter the fact that, as functioning tissues, the tumors changed.

With regard to the "correspondence" noted in the data of table 1 and elsewhere, there is no theoretical reason for believing that conditions like those imposed in these experiments would be a limiting requirement. In this work it might be merely coincidental that "correspondence" in the behavior of the tumors went along with *known* genetic differences. Among the results one can find differences although the recipients differ by only one known gene, but the known genes comprise a very small fraction of the entire genic endowment of the cell and the factors controlling such experiments as these apparently do not lie at known loci, with one possible exception. The exception is the *H-2* locus reported by Snell (*12*), which

is of questionable significance in these experiments because both strain BALB/c and strain DBA/2 carry *H-2*[4], according to Snell. Animals or tissues might correspond in known genes and either correspond or fail to correspond in an adequate number of factors for transplantation, and vice versa. The *H-2* locus just mentioned is a case in point; two strains used corresponded with respect to that gene but apparently did not correspond either for certain known genes or in a sufficient number of factors for transplantation. A somewhat more interesting case has to do with the linkage group in which "brown" is located. Strains BALB/c and DBA/2 are presumably the same at the locus "brown" but in this work there was a difference associated with this linkage group. The difference, which concerned the linkage with "black" pointed out in the preceding section, probably means that the two strains are not alike in certain factors for transplantation that are associated with the linkage group in question. Thus, although it is convenient to speak in terms of the known color genes, what is meant is the associated groups of genes. Furthermore, this does not imply that the effects observed were those of the color gene itself or that the color gene is necessarily an active part of an effective group. It is more probable that the effects observed are the result of the combined action of multiple factors, some of which may be associated with known phenotypes and some of which may not. As a matter of fact, there is no clear evidence that these factors are necessarily a part of either the chromosomes or nucleus. All that can be said is that some of the effects go along with, and are in some way related to, the action of the known color genes that conveniently distinguish the animals used.

In a vein similar to that of the preceding paragraph, one may find, in the data of table 2, evidence of a dissociation between the effects of known genes and the factors controlling transplantation. Following the transplantation of stock tumor it was observed that, like the genes for color, the factors controlling transplantation of the tumor were not uniformly distributed among the segregants whereas, when correspondingly adapted tumor was transplanted, it was found that the factors that controlled its transplantation were, unlike the genes for color, uniformly distributed among the segregants.

It is evident that in relation to transplantation both the transplanted tissue and the recipient host exhibit a high degree of individuality and that genetic constitution exerts a strong influence on the individuality. Accumulated evidence (*9*) makes it clear that both the tissue (at least tumor tissue) and the host may be altered experimentally in this respect. How far this may lead cannot be predicted, but it appears to lead in the direction of a better understanding of what is meant when it is said that the tissue is "foreign" to the host.

Summary

A previous observation, that during growth in a particular F_1 hybrid a tumor acquired an enhanced ability to grow in resistant backcross recipients, was repeated and confirmed.

It was further observed that such enhancement may occur when using a genetically different donor, or recipient, or both.

Although enhancement may be observed following the use of either donor, it was greater in degree when the donor and recipient corresponded with respect to the parent strain foreign to the tumor.

Analysis of the results with respect to the several color segregants in the backcross population lead to the conclusion that some of the factors controlling transplantation in this material were associated with known genes and some were not. The use of "corresponding" and "noncorresponding" donors was followed by qualitatively different results when segregants were considered separately. Thus the specificity involved appears to extend to relatively small genetic differences.

It was concluded that the phenomenon of adaptation in tumor tissue is specific, and that the specificity may be expressed in both a quantitative and qualitative manner.

The mechanism of the change was not revealed although some inferences regarding mechanism may be drawn.

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Complement Fixation in Animal Neoplasia. VI. Development of the Reaction in Various Breeds of Rabbits Carrying the Brown-Pearce Carcinoma[1,2]

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Positive complement fixation reactions in animal neoplasia were obtained by Kidd (*1*) when the sera of certain "blue-cross" hybrid rabbits carrying the Brown-Pearce carcinoma were tested with antigens extracted from the neoplasm. In later reports (*2–6*) Kidd and his co-workers described three types of antibodies that were able to fix complement in combination with normal or neoplastic tissue extracts: 1) A "natural antibody," reacting with fresh extracts of normal and neoplastic tissues, was present in sera from both normal and tumor-bearing rabbits when the sera were inactivated at 56°, but was destroyed when the sera were inactivated at 65° C. The reaction was reduced if the tissue providing the antigen were allowed to extract overnight at 3 to 5° or if the resulting saline extract were stored at 3 to 5° C. 2) A heat-stable (at 65° C.) "induced antibody," also reacting with extracts of normal and neoplastic tissues, appeared in the sera of most market-bought breeds of rabbits after inoculation with the Brown-Pearce carcinoma or with such other neoplasms as the V2 carcinoma. 3) A heat-stable (65° C.) "specific antibody for the distinctive constituent of the Brown-Pearce carcinoma" appeared in high titer in the sera of tumor-bearing rabbits of the "blue-cross," Chocolate-Dutch, and Havana breeds.

Using tumor sera[4] freed of the "natural antibody" by inactivation at 65° C., these workers found that 4 of 17 "blue-cross" (*4*), "most" of 17 Havanas, and "most" of 23 Chocolate-Dutch rabbits (*6*) developed

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[4] The designation "tumor sera" is used in this paper to refer to the sera of rabbits bearing the Brown-Pearce carcinoma.

strongly positive reactions only with extracts of the Brown-Pearce carcinoma; these sera, therefore, contained the "specific antibody" exclusively. In addition, 2 more "blue-cross" and a "few" Havana and Chocolate-Dutch rabbits developed sera which, while reacting to a high titer with Brown-Pearce carcinoma extracts, also reacted, but to a lower titer, with extracts of such normal rabbit tissues as kidney and liver. These latter animals were considered to have developed "induced" as well as "specific antibody." Since none of the animals of these three special breeds developed sera that "reacted as well or better with antigens made from normal kidney and liver tissues than with those derived from neoplasms," the authors assumed that none of these special sera contained "induced antibodies" exclusively. The report of MacKenzie and Kidd (*4*) shows that, in contrast, none of 26 market-bought rabbits (Chinchilla and Agouti) developed "specific antibodies" exclusively, that 5 of the 26 tumor sera reacted to a higher titer with the Brown-Pearce carcinoma extract than with normal kidney extract ("specific and induced antibodies"), and that 10 of the same 26 rabbits developed only "induced antibodies," for their sera reacted to the same or a higher titer with kidney extracts than with extracts of the Brown-Pearce carcinoma. The sera of the 11 remaining rabbits failed to react with either antigen.

We have reported previously (*7*) that when Kidd's 2-hour complement fixation method was followed as closely as possible (tests read at the 100-percent hemolysis endpoint in the presence of two 100-percent units of complement), we obtained very few weakly positive reactions with Brown-Pearce carcinoma extracts and tumor sera from New Zealand White rabbits, the breed available to us at that time. In fact, the only positive reactions obtained were with tumor sera inactivated at 56° or 61°; no positive tests were obtained with sera inactivated at 65° C., the temperature recommended by Kidd for the elimination of the "natural antibody." However, when a quantitative 18-hour method was used with serum inactivated at 61° C., a large proportion (86 percent) of these same New Zealand White animals gave statistically positive reactions that were not given by animals inoculated with a variety of agents and substances other than the Brown-Pearce carcinoma (*8*).

These results indicated that weak reactions were missed when Kidd's less sensitive method was used (shorter fixation time, and the addition to tests and controls of one whole 100-percent unit of complement over and above the one unit required to hemolyze completely the sensitized red cells). It seemed possible, therefore, that the use of a method that apparently detected only very strong reactions might be partly responsible for the reported serological differences between tumor-bearing animals of "special" and of market-bought breeds. This possibility was investigated experimentally in 2 ways: 1)With Brown-Pearce carcinoma extracts as antigens, individual tumor sera from rabbits of 4 market-bought breeds and 1 "special" breed (Havana) were tested by the same 18-hour complement fixation method that we had used previously for testing individual tumor sera from New Zealand White rabbits. 2) The complement fixation

reactions of normal and tumor serum pools from four breeds of rabbits (three market-bought breeds and the Havana breed) were compared by using both our 18-hour method and Kidd's 2-hour method, with normal rabbit kidney and Brown-Pearce carcinoma extracts as antigens.

Tests of Individual Tumor Sera by the 18-Hour Quantitative Technique

Methods

Tumor sera for this study were obtained from market-bought Dutch, Chinchilla, Flemish, and Marten rabbits, as well as from pure-bred animals of the "special" Havana breed. Heavy cell suspensions of the neoplasm were prepared and inoculated in multiple sites as described previously (*7*). For the first series of animals of each breed, the inocula were obtained from New Zealand White rabbits carrying the neoplasm, but the subsequent transfers (about three) into animals of a particular breed were made with neoplastic tissue obtained from animals of the same breed. Palpable tumor usually appeared 1½ to 2 weeks after inoculation. Chinchilla, Dutch, and Flemish rabbits that survived for about 5 weeks were sacrificed, but about half the inoculated Havanas were allowed to live beyond 3 months. It was necessary to sacrifice the Marten rabbits 2 weeks after inoculation, before the experiment was completed, because they were suspected of harboring coccidiosis. Samples of blood for complement fixation tests were obtained by cardiac puncture before inoculation and at weekly intervals thereafter.

Complement fixation tests were performed by the method already described (*7*); the sera were inactivated at 61° C. because we had observed that most specimens tended to show maximum reactivity when inactivated at this temperature. Tests of the sera from animals of each breed were performed with saline antigens that were prepared from pooled neoplastic tissue obtained from tumor-carrying animals of the corresponding breed. This procedure was followed in order to minimize the chances of measuring, besides the reaction between tumor serum and the Brown-Pearce carcinoma antigen, unrelated nonspecific complement fixation due to the admixture of tumor sera from animals of one breed with antigens derived from neoplasms arising in animals of another breed. Since the results of tests of New Zealand White rabbit serum specimens of various serum-antigen ratios had indicated (*8*) that a greater number of positive tests might have been obtained had the sera been tested at dilutions lower than 1:10, the serum samples were tested at two serum-antigen ratios (serum 1:5, antigen 1:40; and serum 1:10, antigen 1:40). All serum specimens from any one animal were tested in one day's run, and usually the tests at both serum-antigen ratios were performed on the same day.

Results of the routine tests were considered to be positive when the titers (number of 50-percent units of complement fixed per ml. of serum) exceeded the highest expected normal titer (*7*, *8*); titers were considered to be in the "high normal" range when they fell within the highest 5 per-

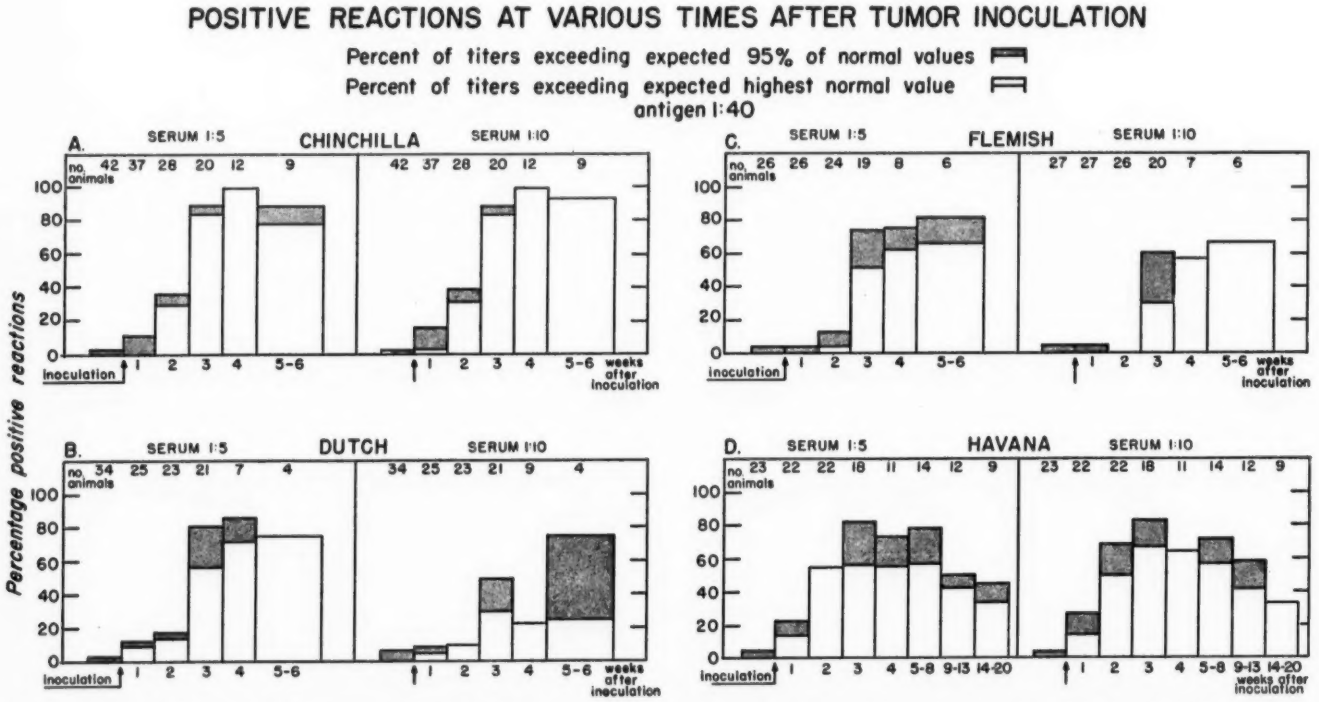
cent of expected normal titers.[5] The expected range of normal titers was calculated for each breed of rabbits, for each antigen, and for each serum-antigen ratio from the results of tests on: 1) the pre-inoculation serum specimens obtained from the animals that later provided the tumor sera for these experiments, and 2) additional individual normal serum specimens from animals that were never inoculated with the neoplasm.

Results

Text-figure 1 presents the precentages of positive reactions given by animals of the four breeds, in which comparable numbers of animals were examined over comparable postinoculation periods. The number of animals from which the percentages were calculated are shown above each percentage bar. The results have been plotted both as the percentages of titers exceeding the lowest 95 percent of expected normal titers, and as the percentages exceeding the expected highest normal values for the particular antigen and serum-antigen ratio used. Thus the highest 5 percent of normal titers is considered to represent the "high normal" range, and titers exceeding the expected highest normal values are considered to be truly positive reactions.

The development of positive reactions in the Chinchilla, Dutch, and Flemish breeds followed the same general pattern as was reported for New Zealand White rabbits (*8*): a few positive reactions appeared during the first 2 weeks, the percentages of positive tests rose sharply during the 3rd week, and they then either increased slightly or remained high during the remaining time that the reaction was followed (through 6 weeks post-inoculation). All of the Chinchilla rabbits that survived had developed positive reactions by the 4th week after inoculation of the neoplasm, with the 2 serum-antigen ratios detecting essentially the same percentages of positive tests. The maximum percentage of positive reactions in the Dutch rabbits was 75, obtained at the 1:5–1:40 ratio in the period 4 to 6 weeks after inoculation. Apparently the higher concentration of serum was necessary to demonstrate many positive reactions in the Dutch rabbits, for at the 1:10–1:40 ratio only 29 percent of positive tests (3 weeks after inoculation) were obtained. The reaction pattern for Flemish rabbits was similar, except that only 1 positive test was noted during the first 2 weeks following inoculation; the maximum percentage of positive tests, 66 at both serum-antigen ratios, was reached in the period 5 to 6 weeks after inoculation. The 1:5–1:40 ratio appeared to be more sensitive than the 1:10–1:40 ratio for detecting positive reactions in the first 4 weeks after inoculation of the Flemish rabbits. The proportion of 2-week-post-inoculation Marten sera giving positive reactions (30 percent) was similar to the proportions found in the other breeds at the corresponding postinoculation time period. No positive reactions were obtained with the sera

[5] The highest expected normal titer was calculated to be the mean value of normal titers plus 3 Standard Deviations, and the point separating the lowest 95 percent of expected normal titers from the highest 5 percent ("high normal" range) was taken to be the mean value plus 1.645 S. D.



TEXT-FIGURE 1.—Percentages of positive reactions obtained at various times after inoculation of the neoplasm into four breeds of rabbits.

from 6 uninoculated Marten rabbits that were heavily infected with coccidiosis.

Our previous studies with New Zealand White rabbits indicated that at the usual serum-antigen ratio used, few tumor sera were so reactive that they fixed more than five 50-percent units of complement (1:10–1:40 titer of 125); the highest 1:10–1:40 titer encountered among 190 animals of this breed was 220. Among the four breeds of animals studied here (no more than 42 animals of any one breed), the number of animals of each breed that developed titers of 125 or more, and the respective maximum titers attained, were as follows: Dutch, 2 and 425; Chinchilla, 7 and 193; Flemish, 3 and 164; and Havana, 1 and 168, respectively. Although we found that, as Casey (*9*) had stated, Havana rabbits resisted extensive growth of the Brown-Pearce carcinoma, it is obvious from the above enumeration that the pure-bred Havana rabbits used in our studies did not develop the exceptionally reactive tumor sera that Kalfayan and Kidd (*6*) reported.

Comparisons of the 18-Hour and 2-Hour Methods

Methods

Normal and tumor serum pools [6] from four breeds of rabbits (New Zealand White, Chinchilla, Dutch, and Havana) were tested simultaneously by our usual 50-percent hemolysis procedure with a fixation period of 18 hours, and by the 2-hour method described by Kidd (*1*, *4*).[7] The tests performed by Kidd's 2-hour procedure were carried out as he described,[7] but in addition similar tests and controls were set up using progressively smaller amounts of complement, as is done in our 18-hour procedure, so that the exact amount of complement required for 50-percent hemolysis could be determined for each test and control. Samples of the serum pools were inactivated for 30 minutes at 56°, the temperature at which Kidd's "natural antibody" was demonstrated; at 61°, the temperature we had chosen for the 18-hour test; and at 65° C., the temperature chosen by Kidd for his tests. The sera were then diluted serially from 1:10 to 1:40 or 1:80 for testing. The serum pools from each breed of rabbit were tested with 1:40 dilutions of a saline extract of Brown-Pearce carcinoma tissue (tumor antigen) and with the same dilution of a saline extract of normal kidney tissue (kidney antigen), both obtained from the corresponding breed.

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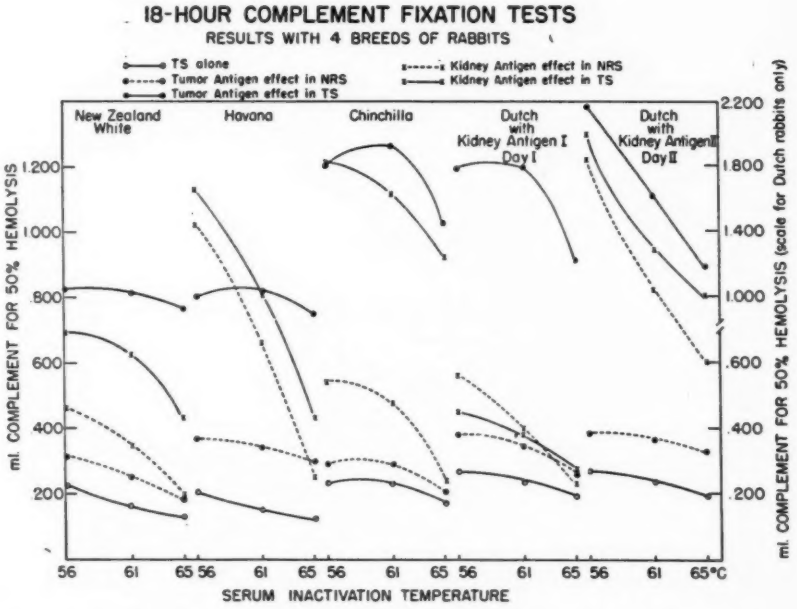
[6] Normal serum pools are designated as NRS, tumor serum pools as TS. The TS pool from each breed contained the most reactive individual tumor serum specimens (about 4 weeks after inoculation) from the animals that developed positive reactions.

[7] To saline (0.85 percent NaCl) dilutions of serum were added two 100-percent hemolysis units of complement and an antigen consisting of a partially clarified saline extract of either normal rabbit kidney or the neoplasm. Serum, antigen, and hemolytic system controls were also included. After incubation for 2 hours at room temperature, remaining complement was estimated visually from the degree of hemolysis resulting from the use of the hemolytic indicator— 5 percent sheep cells sensitized with four 100-percent units of hemolysin and incubation for 30 minutes at 37° C. The amounts of fixation were correlated with the degrees of hemolysis as follows: 0 percent hemolysis indicated 4-plus fixation; about 25 percent hemolysis, 3-plus; about 50 percent, 2-plus; about 75 percent, 1-plus; about 90 percent, plus-minus; and 100 percent, negative.

Results

In text-figure 2 are presented the results of the 18-hour quantitative tests with 1:10 dilutions of TS and NRS from each of the four breeds. The data for two series of comparative tests with the Dutch NRS and TS (for day I and day II) are included; the same sera and the same preparation of Dutch tumor antigen were tested on both days, but for each day's series a different preparation of kidney antigen was used.



TEXT-FIGURE 2.—Comparison of the effects of tumor antigen and of kidney antigen in tumor and normal serum pools from four breeds of rabbits. Tests read at 50-percent hemolysis endpoint after 18-hour fixation period. Sera diluted 1:10, antigens 1:40.

For each breed the ml. of 1:100 complement required for 50 percent hemolysis of the sensitized red cells in the indicator system was determined in each of the following titrations:

NRS=normal rabbit serum pool
NRS+A=normal rabbit serum pool plus antigen (tumor or kidney)
TS=tumor serum pool
TS+A=tumor serum pool plus antigen (tumor or kidney)

The value TS+A thus represents the complement requirement in the presence of TS plus the *additional* requirement due to the presence of antigen. Complement was considered to have been fixed when the presence of antigen in TS caused a greater *additional* requirement for complement than did the presence of antigen in NRS.

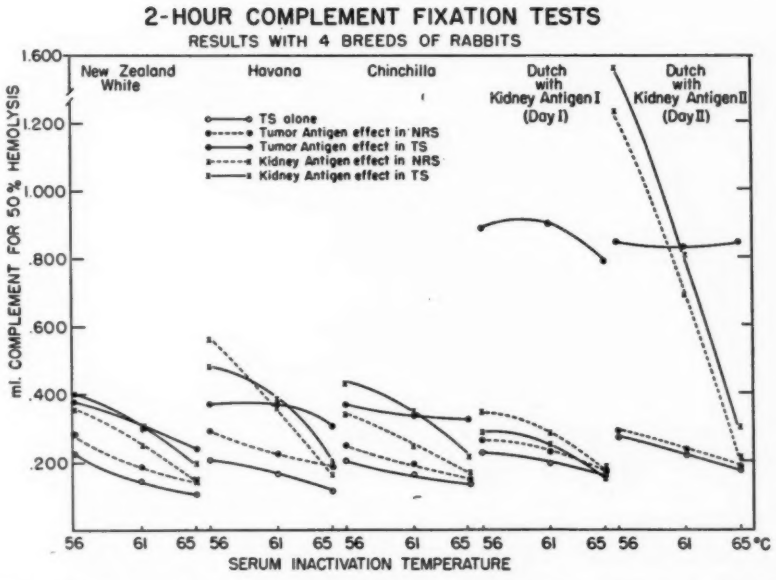
Text-figure 2 shows the base-line amounts of complement required in the TS titrations, and above these curves have been plotted the *additional* complement requirements due to the presence of tumor or kidney antigen in normal and in tumor serum inactivated at 56°, 61°, and 65° C. The results have been plotted in terms of the

amount of complement required for 50-percent hemolysis in order to show the effects of each antigen in NRS as well as in TS. However, the amounts of complement actually fixed by each TS pool are indicated by the distances between the dotted line (effect in NRS) and the solid line (effect in TS) for kidney or for tumor antigen. If the solid line is *below* the dotted line (for example, Dutch serum pools inactivated at 56° and 61° with kidney antigen I) no complement was fixed, for the antigen effect in TS *did not* exceed that in NRS.

The results of each of these series of tests show that the comparatively high kidney-antigen effects in the presence of NRS inactivated at 56° C. were markedly reduced as the NRS pools were inactivated at higher temperatures, while the low tumor-antigen effects in the same NRS pools remained essentially the same throughout the range of inactivation temperatures used. The effect of Dutch kidney antigen II was much greater than that of Dutch kidney antigen I, but increasing the temperature at which the Dutch serum pools were inactivated resulted in the same proportional decrease in the antigen effects of both preparations. With all breeds, the curves representing the kidney-antigen effects in TS tended to parallel those representing the kidney-antigen effects in NRS, and with one exception [8] the curves showing the tumor-antigen effects in TS more closely resembled those showing the corresponding effects in NRS. Each of the four TS pools fixed some complement with its respective kidney antigen (except that Dutch TS fixed complement only with Dutch kidney antigen II), but in each case the amount fixed with kidney antigen was definitely less than the amount fixed with tumor antigen.

Text-figure 3 shows the results of similar 2-hour tests on the same sera and antigens, but performed with the reagents Kidd prescribed, and read at the 50-percent hemolysis endpoint. This quantitative evaluation of the results of tests performed by Kidd's method made it possible to measure precisely the weaker antigen effects that would be missed if the tests were read only as he described, at the 100-percent hemolysis endpoint in the presence of two 100-percent units of complement. The data have been plotted in the same manner as were the results of the 18-hour tests shown in text-figure 2. With all breeds, much less complement was fixed in 2 hours than in 18 hours, and the effects of the antigens in the NRS pools were similarly diminished. However, in all cases the curves representing the 2-hour kidney-antigen effects in NRS had the same characteristic shapes as did those representing the 18-hour effects. Also, as in the 18-hour tests, the amount of complement fixed in 2 hours with TS and tumor antigen was in each case more than the amount fixed with kidney antigen. Thus, quantitative evaluation of the results of the 2-hour tests showed that the technical differences between Kidd's method and ours (composition of the diluent, sheep red-cell concentration, hemolysin concentration, and fixation time) made little difference in either the comparisons between tumor-antigen and kidney-antigen effects or the comparisons among the four breeds. The use of the 2-hour test showed only a diminution of most of the effects of antigen and serum.

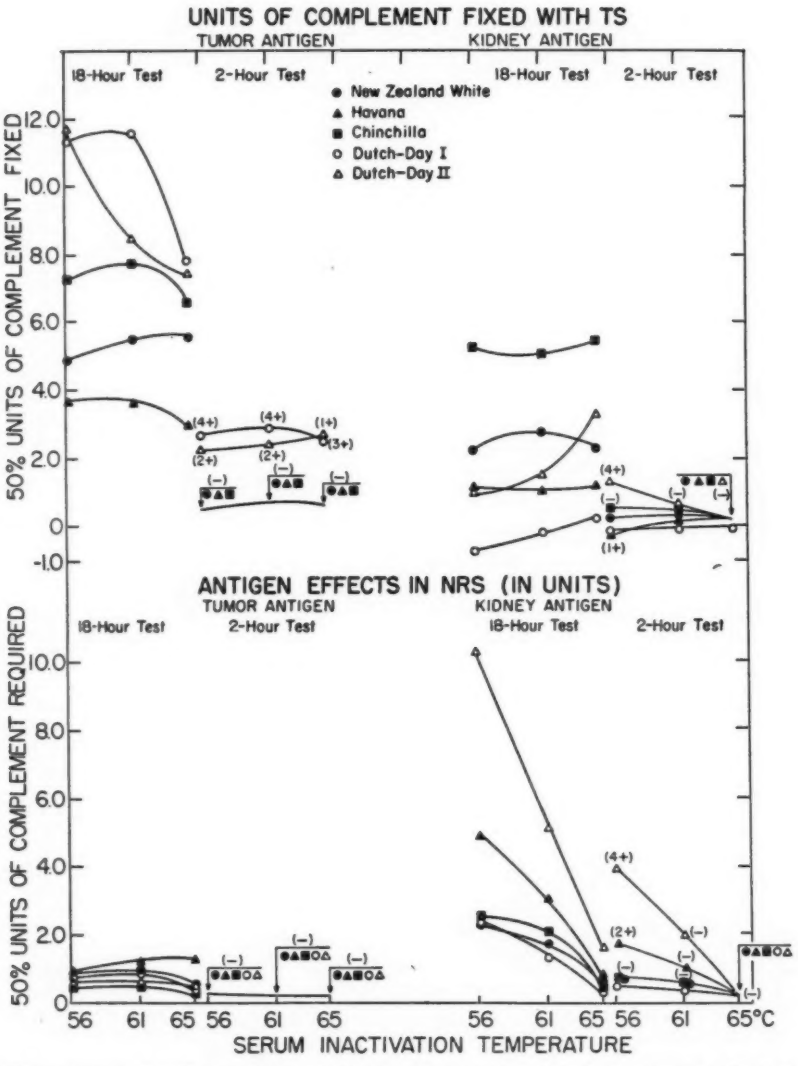
[8] On days I and II the Dutch tumor-antigen effect with the Dutch TS inactivated at 56° C. differed considerably; therefore, the TS-plus-tumor-antigen curve accompanying the results with Dutch kidney antigen II did not resemble the corresponding tumor-antigen curve in the presence of NRS in the same series of tests.



TEXT-FIGURE 3.—Comparison of the effects of tumor antigens and of kidney antigens in tumor and normal serum pools from four breeds of rabbits. Tests read at 50-percent hemolysis endpoint after 2-hour fixation period. Sera diluted 1:10, antigens 1:40.

The data shown in text-figures 2 and 3 have been used to calculate the 50-percent units of complement actually fixed in the 18-hour and 2-hour tests; in text-figure 4 the units fixed with kidney and tumor antigens and 1:10 dilutions of the four TS pools have been plotted against the serum inactivation temperatures. Also shown are the effects of kidney and tumor antigens in the presence of NRS. The units fixed were calculated by dividing the ml. of 1:100 complement fixed by the ml. of 1:100 complement required for 50-percent hemolysis in an immediate complement titration, performed the day the tests were set up. The antigen effects in NRS, as ml. of 1:100 complement, were divided by the same number to obtain the values in terms of 50-percent complement units, for comparison with the units fixed in the tests proper (in the presence of TS).

The degrees of fixation in the 2-hour tests were measured not only in terms of 50-percent units of complement (the plotted points) but also by the less sensitive visual method used by Kidd, in terms of the total inhibition of hemolysis, expressed in plus signs, in tubes carrying two 100-percent units of complement (see footnote 7). The extent of inhibition of hemolysis in this test is dependent upon the sum of the effect of serum, the effect of antigen, and the amount of complement actually fixed. Incomplete hemolysis, as a result of any combination of these factors, would be interpreted as representing a "positive" test, or true complement fixation. The results of these visual estimations of the extent of hemolysis



TEXT-FIGURE 4.—Comparisons of 1) the 50-percent units of complement fixed in 2- and 18-hour tests with tumor and kidney antigens; 2) the 2- and 18-hour antigen effects in NRS (in 50-percent units); 3) the results of 2-hour tests read visually, in plus signs; (−), (1+), (2+), etc, represent visual estimates of inhibition of hemolysis in tubes containing two 100-percent units of complement—Kidd test.

inhibition are indicated in text-figure 4 by the plus and minus signs beside the plots of the number of 50-percent units of complement fixed in the 2-hour tests.

Curves obtained from results of the tests with tumor antigens show that the TS pool from market-bought Dutch rabbits fixed the greatest amount

of complement in both 2 and 18 hours and gave, in the 2-hour tests, the only "positive" tests when read by Kidd's visual method. The "positive" 2-hour Kidd tests with Dutch TS inactivated at 65° C. (obtained on both day I and day II) were the only "positive" tests obtained with tumor serum inactivated at the temperature recommended by Kidd. Results with the 18-hour procedure showed that the least amount of complement was fixed by the TS pool from Havanas, reported (*6*) to develop much "specific antibody" (high titers with tumor antigen). Actually, the TS pools from the four breeds showed no more variation in 18-hour reactivity than did the individual New Zealand White tumor sera tested previously (*8*).

The 2-hour tumor antigen effects in NRS were practically nil (0.2 50-percent unit) when measured quantitatively, and visual readings of these tests were also negative, even when the NRS pools were inactivated at 56° C. Apparently, as Kidd and Friedewald (*2*) predicted, the tumor antigens prepared by overnight extraction with saline did not contain enough "natural tissue constituent" to react with NRS in the 2-hour tests. The small 18-hour tumor-antigen effects in NRS (1.2 50-percent units or less) did not appear to represent true reactions, for these studies as well as those previously reported (*7*) have shown that the tumor-antigen effect in the presence of NRS was not reduced by further dilution of the NRS, as would be expected if a serological reaction actually took place.

From text-figure 4 it can be seen that, in spite of the large kidney-antigen effects in NRS, measurable amounts of complement were actually fixed in the 18-hour tests of all four TS pools with kidney antigens; however, in each case these amounts were less than the amounts of complement fixed with tumor antigens. In 2 hours the most reactive TS (Dutch) fixed only 1.2 units of complement with kidney antigen, and the other TS pools fixed even less (no complement was fixed by the Havana TS). The "positive" Kidd tests with Havana and Dutch TS could not be considered true reactions, because the control tests with the corresponding NRS pools were also "positive." If the 18-hour reactivity with kidney antigen can be considered to be due to the "induced antibody" described by Friedewald and Kidd (*3*), then all four breeds developed both "specific" and "induced antibodies"—"specific" because more complement was fixed with tumor than with kidney antigens, and "induced" because some complement was definitely fixed with kidney antigens. The response of the Havana rabbits was qualitatively and quantitatively no different from the responses of the 3 market-bought breeds.

The effects of kidney antigens in NRS pools inactivated at 56° and 61° C. were high, even in 2 hours; in fact, with Dutch and Havana NRS pools inactivated at 56° C. they were sufficiently high to give incomplete hemolysis in the presence of two 100-percent units of complement. In contrast with the tumor-antigen effects in NRS, the kidney-antigen effects in all four NRS pools inactivated at 56° and 61° C. were definitely reduced as the sera were diluted. Reduction of the antigen effect with reduction of the serum concentration is characteristic of true reactions in general, and it

was demonstrated consistently in tests of the serially diluted TS pools with both kidney and tumor antigens. Thus, although incomplete hemolysis ("positive" controls with Dutch and Havana NRS) in the 2-hour Kidd test does not necessarily indicate a true reaction with a "natural antibody," the quantitative method showed at least part of the kidney-antigen effect in NRS was due to some type of a true reaction, even in the case of the NRS pools that showed complete hemolysis in the Kidd tests (Chinchilla and New Zealand White NRS pools). Apparently even when the kidney antigens were prepared by overnight extraction, they contained "normal tissue constituent" (*2*) which reacted with the "natural antibody" in normal rabbit serum.

Discussion

We have concluded that the results of these comparative tests showed no distinct qualitative differences among the four breeds of rabbits in their reactions with the two types of antigens. In both the 18-hour and the 2-hour quantitative tests (evaluated in terms of the number of 50-percent units of complement fixed) the Havana TS pool did not show the high content of "specific antibody" reported by Kalfayan and Kidd (*6*), for it fixed only a moderate amount of complement with tumor antigen and it did react, though weakly, in 18-hour tests with kidney antigen.

Our original premise, that the use of Kidd's less sensitive method might be responsible for the reported serological differences between tumor-bearing animals of "special" and of market-bought breeds, was not substantiated, for the results obtained by Kidd's method did not show that the pool of "special" Havana tumor sera differed from the pools of TS obtained from market-bought breeds. It is conceivable, though, that a more reactive Havana TS pool, inactivated at 65° C. as Kidd recommended, might have required enough complement in combination with tumor antigen to give a "positive" 2-hour Kidd test without requiring enough complement to give a "positive" Kidd test with kidney antigen. This possibility is indicated by the 2-hour results of the Kidd test with Dutch TS and tumor antigen. Actually, the fact that the highly reactive Dutch TS inactivated at 65° C. did give a "positive" Kidd test might have been taken as an indication that the Dutch TS contained only the "specific antibody" reacting with tumor antigen; yet, as text-figure 4 shows, the Dutch TS inactivated at 65° C. fixed over 3 units of complement with kidney antigen in the more sensitive 18-hour test. Our failure to demonstrate a serological distinction between the TS pools from Havanas and the TS pools from market-bought breeds may also have been due to the genetic constitution of the Havana rabbits that we used, or to the fact that we tested Havana TS only with antigen obtained from normal or neoplastic Havana tissues.

The high kidney-antigen effects in NRS inactivated at 56° and 61° C., and the fact that they were reduced as the NRS pools were further diluted, were considered to be evidence that actual reactions took place between normal rabbit serum and kidney extracts. This conclusion agrees with the report of Kidd and Friedewald (*2*), even though the

kidney antigens that we used (prepared by overnight extraction with saline) were not "fresh" as defined by these workers (extracts prepared and used on the same day). The tumor antigens that we used were also prepared by overnight extraction; perhaps, as Kidd and Friedewald indicated, the Brown-Pearce carcinoma tissue contains only a small amount of the "normal tissue constituent," which disappeared during overnight extraction, for neither in our previous experiments nor in those reported in this paper was there any evidence that tumor antigens reacted with NRS. The antigens used in our experiments (tumor or kidney) were never subjected to the other procedures reported (*2*) to destroy or greatly reduce the "normal tissue constituent" (storage of tissue in glycerol, heating the saline antigen to 56° C. for 30 minutes before use, storing the saline antigen at refrigerator temperature after the initial extraction in the refrigerator).

Maltaner's report (*10*) of positive complement fixation tests (quantitative procedure, read at the 50-percent hemolysis endpoint) with normal rabbit serum inactivated at 56° C. and fresh extracts of rabbit kidney agree in some respects with the results reported here. He had shown previously (*11*, *12*) that substances with high thromboplastic activity, such as cephalin, inhibited complementary activity, and that the thromboplastic activity was enhanced by normal rabbit serum even after it had been inactivated at 56° C. for 30 minutes. Fresh extracts of rabbit kidney were also found to possess thromboplastic activity, and the enhancement of this activity (reduction of the time required to clot recalcified rabbit plasma) by various dilutions of normal rabbit serum inactivated at 56° C. paralleled the amount of complement fixed (or inhibition of complementary activity) in tests with the same dilutions of normal rabbit serum inactivated at 56° C. and fresh extracts of rabbit kidney as antigen. Maltaner also reported a parallel diminution of the number of 50-percent complement units fixed and the enhancement of clotting activity as the inactivation temperature of the normal rabbit serum was increased (56°, 60° and 65° C.). He concluded that certain antigens, such as fresh rabbit kidney extracts, which possessed thromboplastic activity may give "nonspecific" complement fixation tests with serum that enhances such activity, such as normal rabbit serum inactivated at 56° C.

While we have not investigated the thromboplastic activity of the kidney antigens that we used, their reactivity with the NRS pools may have been a demonstration of the phenomenon reported by Maltaner. If so, the reactivity of kidney antigen with NRS is not so labile that it disappears when the kidney tissue is extracted overnight in the refrigerator or upon storage of the extract at about −20° C., for the kidney antigens that we used were stored in the frozen state for several months before use. Since we could not demonstrate that the tumor antigens actually reacted with NRS inactivated at any of the 3 temperatures, it also follows that the tumor antigens we tested did not give the "nonspecific" reactions described by Maltaner.

Summary and Conclusions

The complement fixation reactions of sera obtained from rabbits bearing the Brown-Pearce carcinoma have been studied in animals of the Chinchilla, Dutch, Flemish, Marten, and Havana breeds.

Results of tests performed by our usual 18-hour quantitative method (using extracts of the neoplasm as antigens) showed that positive complement fixation reactions developed in the animals of each of these breeds according to the same general pattern as has been reported previously for a large group (190) of New Zealand White rabbits. The maximum percentages of positive tests obtained for the smaller groups of animals of the various breeds are as follows: 100 in Chinchillas at 4 weeks, 75 in Dutch at 4 to 6 weeks, 66 in Flemish at 5 to 6 weeks, and 67 in Havanas at 3 to 8 weeks after inoculation. Postinoculation sera from the pure-bred Havana rabbits used in these studies were no more reactive than were similar sera from the market-bought breeds.

Our usual quantitative 18-hour complement fixation procedure was compared with the 2-hour method described by Kidd in tests of sera from four breeds of rabbits bearing the Brown-Pearce carcinoma (3 market-bought breeds, Chinchilla, Dutch, and New Zealand White; and 1 "special" breed, the Havana, reported to develop serum that was highly reactive only with extracts of the neoplasm). When the sera were tested with normal kidney extracts and extracts of the Brown-Pearce carcinoma as antigens, no qualitative differences among the reactions of the four breeds were noted in tests performed by either complement fixation technique. Inoculation of the Brown-Pearce carcinoma into the pure-bred Havana rabbits did not elicit a specific serological response that differed in character from the responses elicited in the 3 market-bought breeds.

A serological distinction was observed between the normal rabbit kidney antigens and the Brown-Pearce carcinoma antigens: 1) normal rabbit serum pools from all four breeds appeared to react with extracts of normal kidney tissue but not with extracts of the Brown-Pearce carcinoma; and 2) extracts of the Brown-Pearce carcinoma showed greater reactivity with the sera of rabbits carrying the neoplasm than did extracts of normal kidney. These differences between normal kidney extracts and Brown-Pearce carcinoma extracts have been interpreted to mean that there was a greater concentration of the specific active component responsible for the complement fixation reaction in extracts of the Brown-Pearce carcinoma than in extracts of normal rabbit kidney tissue.

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Studies of the Carcinogenic Effect of Urethan on Transplanted Lung Tissue in Strain A Mice[1]

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Carcinogens, in addition to their tumor-inducing properties, have also been shown to produce a number of systemic effects in treated animals. Lorenz and Stewart have reported anasarca, atrophy of the genital tissue, and atrophy of the lymphoid and myeloid tissues following the oral administration of methylcholanthrene and 1,2,5,6-dibenzanthracene to mice (*1*). The carcinogenic hydrocarbons have also been shown to produce progressive weight loss in rats (*2, 3*). Urethan, used in the present study, depresses bone-marrow enzymes (*4*), interferes with mitotic activity (*5*), produces a leukopenia (*6*) and thrombocytopenia (*7*). More recently it has been shown that antibody titers are depressed in mice treated with a number of carcinogens (*8*). By what mechanism or mechanisms the carcinogens produce these systemic effects and promote tumor development is obscure, and whether the two phenomena represent separate reactions or are mutually dependent is not clear. It is quite certain, however, as shown by Heston and Dunn (*9*), Huseby and Bittner (*10*), and Shapiro and Kirschbaum (*11*), that the tissue itself must be susceptible before cancerous changes are induced. However, whether the carcinogen acts directly on the tissue, or indirectly on the tissue as a result of systemic alteration, or by both mechanisms is not as readily apparent. The importance of a systemic influence was suggested by Heston and Dunn when they found an increase in the expected tumor incidence in lungs of strain L mice transplanted into LAF_1 hybrids, and a decrease in the expected tumor incidence in lungs of strain A mice transplanted into LAF_1 hybrids, when the host was treated with 1,2,5,6-dibenzanthracene after transplantation (*9*).

In the present work, the carcinogen urethan was administered to strain A mice in an attempt to clarify the relationship, if any, between its systemic and carcinogenic effects.

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Materials and Methods

Male and female strain A mice of the Heston subline, 6 to 8 weeks old, were used both as donors of, and hosts for, the lung tissue transplants. Each of 64 mice received one intraperitoneal injection of urethan, 1 mg. per gram of body weight. Twenty-four hours later 4 of these mice were sacrificed and their lungs were removed. The lungs from each animal were divided into approximately 30 pieces and transplanted with a 12-gauge trocar subcutaneously into the right axilla of the remaining 60 urethan-treated animals, and into the right axilla of 60 untreated strain A mice. At the same time lung tissue from 4 untreated strain A mice was prepared in the same manner and transplanted into the left axilla of both of the above groups. In making these transplants, lung tissue from each of the donor mice was divided among the various groups of test animals so that no single mouse contributed more heavily to one group than to another. For clarity the experimental animals may be labeled:

1. Urethan-treated lung in urethan-treated animals
2. Untreated lung in urethan-treated animals
3. Urethan-treated lung in untreated animals
4. Untreated lung in untreated animals

Six months after transplantation, the 7- to 7½-month-old mice were sacrificed and their lungs were removed and examined for lung tumors under a dissecting microscope. At the same time the transplanted lung tissue was recovered and placed in Tellyesniczky's fixative. The fixed specimens were embedded in paraffin, sectioned serially, and stained with hematoxylin and eosin.

Histology of Lung Transplants

The histology of lung transplants in carcinogen-treated animals has been previously described (*9*). The same classification has been used in the present study for the tumors and non-neoplastic reactions.

Necrosis and severe inflammatory reaction in the transplants were exceptionally rare. In some transplants the bronchi were dilated and filled with leukocytes. The lymphocytic infiltration varied and was in some transplants so extreme as to make the tissue resemble lymphatic tissue. The results of the study of the epithelial changes are presented in table 1, where the transplants are grouped according to treatment. By a *pulmonary tumor* (fig. 1) is meant an unquestionable tumor, similar to that occurring spontaneously in mice. This tumor has a distinct morphology, of which there are many excellent descriptions. Thirty-five tumors were found in the 215 transplants and the tumors were usually single. In only 4 transplants was more than one tumor found, and in these 4 cases no more than 2 tumors arose per transplant. The size of the tumors varied from small, 5 × 5 microns, to large macroscopically visible nodules 60 × 70 microns. In addition to these distinct tumors a *tumorlike lesion* (fig. 2) was found in 5 transplants. These lesions did not extend beyond the boundary of the transplants, and the alveolar walls showed an

increased cellularity and some cellular alteration. The cells were round, sometimes cuboidal or polygonal, and the cytoplasm was acidophilic and pale. The nuclei were large, spherical, and contained one to two nucleoli. Mitotic figures were occasionally seen. In general, the histologic picture was that of the peripheral portion of a fully developed tumor.

Cases grouped under the heading *"foci of increased cellularity"* form a group that must be considered with some reservation. The alveolar cells in these transplants were slightly more prominent and more numerous (fig. 3) in focal areas. Large cells measuring 15 to 20 microns were found, and some of their nuclei were hyperchromatic. The cytoplasm was acidophilic.

Bronchial proliferation, which was marked in 67 transplants, was usually easily distinguished from the changes described above. Differential characteristics proposed by Heston and Dunn (*9*) were used where the distinction was more difficult.

Proliferation of squamous epithelium with occasional keratinization was observed in 8 cases (fig. 4).

Histogenesis of the Pulmonary Tumors

Inflammation has been thought to have some effect on the development of pulmonary tumors. In this material, however, the incidence of inflammatory reaction was significantly less in tumor-bearing lung transplants than in transplants without any sign of epithelial activity. On that basis it is believed that inflammation played no part in the development of these tumors. This is also in agreement with more recent studies (*12*). No connection could be found between the incidence of bronchial proliferation and that of tumor development. In three cases extension of the tumor into bronchi was found and in one case there was an involvement of the bronchioles and bronchi. This was believed to be a secondary extension of the tumor.

The tumors were not encapsulated. The alveoli around the tumors were often lined by a tumorlike epithelium, and foci of larger, round or cuboidal alveolar cells with eosinophilic cytoplasm were observed. This suggests, as pointed out by Mostofi and Larsen (*12*), that the increase in size of the tumor might be due in part to the development of new tumor cells in the periphery of the tumor. In fact, the histology of the peripheral part of most tumors was the same as the lesion called "tumorlike lesion" in 5 transplants, and was also similar to the epithelial changes called "foci of increased cellularity" found in 35 lung transplants. The histologic study of these lesions strongly indicates that the tumors arise from alveolar cells, thus supporting the recent idea of the origin of these tumors (*12*).

When comparing the different experimental groups, the group of transplants in which foci of increased cellularity were found must be regarded, as already mentioned, with some reservation. However, it is a very interesting group from the point of view of the histogenesis of pulmonary tumors. Between this lesion and fully developed tumors there was a link formed by

TABLE 1.—*Incidence of tumors and other cellular changes in hosts and transplanted lung tissue*

Host	Host lungs				Lung transplants							
	Number of mice	Number with tumors	Percent with tumors	Average number of tumors per lung	Treatment	Number of transplants	Number of tumors	Percent with tumors	Number with tumor-like changes	Number with foci of increased cellularity	Number with bronchial proliferation	Number with squamous epithelium
Urethan-treated	58	58	100	28.8	Urethan	54	22	40.74	1	4	13	2
					Untreated	56	1	1.78	1	12	19	5
Untreated	55	17	30.9	0.34	Urethan	52	11	21.15	3	9	14	0
					Untreated	53	1	1.92	0	10	21	1

tumorlike changes, and all the different gradations were found. The changes were also similar to the early changes described by Mostofi and Larsen (*12*) in their study of the histogenesis of pulmonary tumors. It may be of interest that these presumably early lesions seemed to be more common in the experimental groups where tumors were rare. This fact, if significant, may suggest that *a*) these changes are not connected with tumor development, but might also point to the direction that *b*) in the groups where most tumors were found these early changes had already progressed to a tumor, and thus only a few transplants were found with epithelial changes but no tumor.

Incidence of Lung Tumors

The results summarized in table 1 show the expected 100-percent tumor incidence in the lungs of host, urethan-treated strain A mice at 7 months of age. The 30.9-percent lung-tumor incidence observed in the untreated host animals of the same age is also similar to the incidence previously noted in this laboratory in other untreated animals of the same strain and age. The average number of tumors per mouse was 28.8 for the treated group and 0.34 for the untreated group.

As shown in table 1, the transplanted pieces of lung tissue from untreated animals developed approximately the same number of tumors in both the treated and untreated host. A comparison of lung transplants from treated animals, however, shows the development of almost twice as many tumors in the treated as in the untreated hosts. By applying the chi square test this difference is shown to be significant at the 3 percent level (P=between 0.02 and 0.03).

There was no correlation between the number of tumors developed in the host lungs and the number of tumors developed in the transplanted lungs in any of the animals, nor did the sex seem to influence the host or transplanted tumor incidence. Further, it was observed that the total tumor incidence in the transplants in each group resulted from an approximately equal number of tumors contributed by each donor.

Discussion

Urethan was selected as the carcinogen in these experiments because, in addition to its carcinogenic and systemic effects, its rate of excretion is rapid. Studies of C^{14}-labeled urethan indicate that 24 hours after administration 92.7 percent of the urethan is eliminated (*13*). Consequently, it was expected that the small residual amount of urethan would have a negligible direct effect on lung tissue transplanted 24 hours after treatment of the host, and also it would seem that any tumors which might develop in untreated lung tissue transplants would be the result of a systemic change in the host. Therefore, if the effect of a systemic change was of importance in carcinogenesis, the highest tumor incidence

would be expected in urethan-treated and untreated tissue transplanted to a urethan-treated host, and the incidence might be expected to be approximately equal. On the other hand, if malignancy results only from a direct combination of tissue and a carcinogen, the highest tumor incidence would be expected in urethan-treated transplants in either treated or untreated hosts and again the incidence might be expected to be about equal.

As seen in table 1 however, no clear delineation can be made along these lines, but rather the importance of the 24-hour period immediately following the injection of urethan is evident.

The assumption that the 7.3 percent of residual urethan is not in itself sufficient to induce tumors seems correct in light of the tumor incidence of untreated tissue, which was 1.78 percent in treated hosts and 1.92 percent in untreated hosts. Further, it would appear that this small amount of residual urethan can be discounted when comparing the 40.74 percent tumor incidence of treated tissue in a treated host with the 21.15 percent tumor incidence in an untreated host because, as Henshaw has reported, 2 injections of urethan each equal to the single dose used in the present work were needed to double the tumor incidence (*14*).

It would seem that the action of urethan is confined to the 24-hour period following its injection, although the nature of the reaction is less easily ascertained. This seems particularly important since it indicates the relatively short duration of exposure of a susceptible tissue to a carcinogen (urethan) required to ultimately produce a tumor.

Since the urethan-treated transplants showed a higher tumor incidence than the untreated transplants, the immediate conclusion might be that a direct effect of the carcinogen on susceptible tissue in the first 24 hours after treatment is the main factor in urethan carcinogenesis. Undoubtedly a change does occur in the tissue, but whether it can be concluded to be the result of a direct combination with the carcinogen seems less certain when the fate of urethan-treated tissue in treated and untreated hosts is compared. In this case, the doubled tumor incidence of the treated transplants in treated hosts over the treated transplants in untreated hosts suggests that an alteration also occurs in the animal that makes it a more favorable site for tumor development.

The transplantation process *per se* appears to inhibit tumor development, since the tumor incidence in the treated transplants in treated hosts was approximately 40 percent. On the basis of 30 tumors per animal in the host the expected tumor incidence would be about 1 tumor per transplant or nearly 100 percent. These findings are in agreement with the observations of Heston and Dunn (*9*).

This inhibition of tumors by the transplanting process might be great enough to mask the effect of systemic change in the untreated transplants but it would not explain the difference noted when urethan-treated tissue transplanted to a treated host produced twice the number of tumors observed in identical tissue in an untreated host.

Summary

Data has been presented on the effect of urethan on lung tissue of strain A mice transplanted subcutaneously into urethan-treated and untreated strain A mice.

The carcinogenic effect of urethan is largely confined to the first 24 hours after injection, since lung tissue taken from urethan-treated animals 24 hours after treatment and transplanted to untreated animals developed tumors in 22 percent of the cases. It would also appear that the carcinogenic effect of urethan on transplanted strain A lung tissue from untreated animals is absent 24 hours after urethan administration to the host. There persists, however, 24 hours after urethan administration, some factor that enhances the tumor incidence in urethan-treated lung tissue transplanted into hosts previously treated with urethan over the incidence in the same tissue transplanted into untreated animals.

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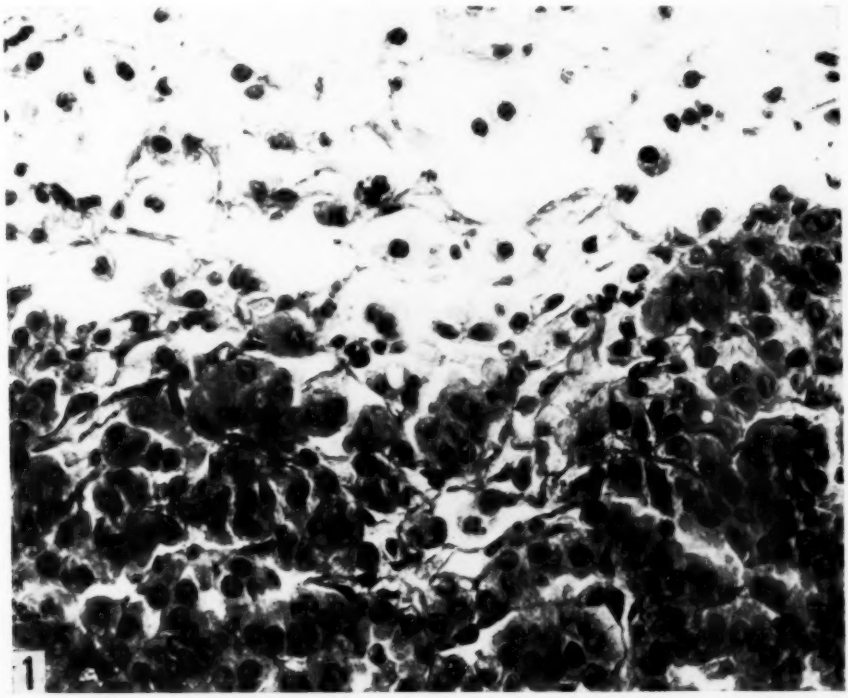
PLATE 31

Changes observed in the transplanted lung tissues:

FIGURE 1.—A fully developed pulmonary tumor. At the periphery of the tumor, neoplastic cells line pre-existing alveoli. Hematoxylin and eosin. × 500

FIGURE 2.—Alveolar cell proliferation, a tumorlike change. The alveolar cells are increased in number, the cytoplasm is acidophilic and the nuclei are large. Large macrophages filled with brown pigment are seen at the right. Hematoxylin and eosin. × 500



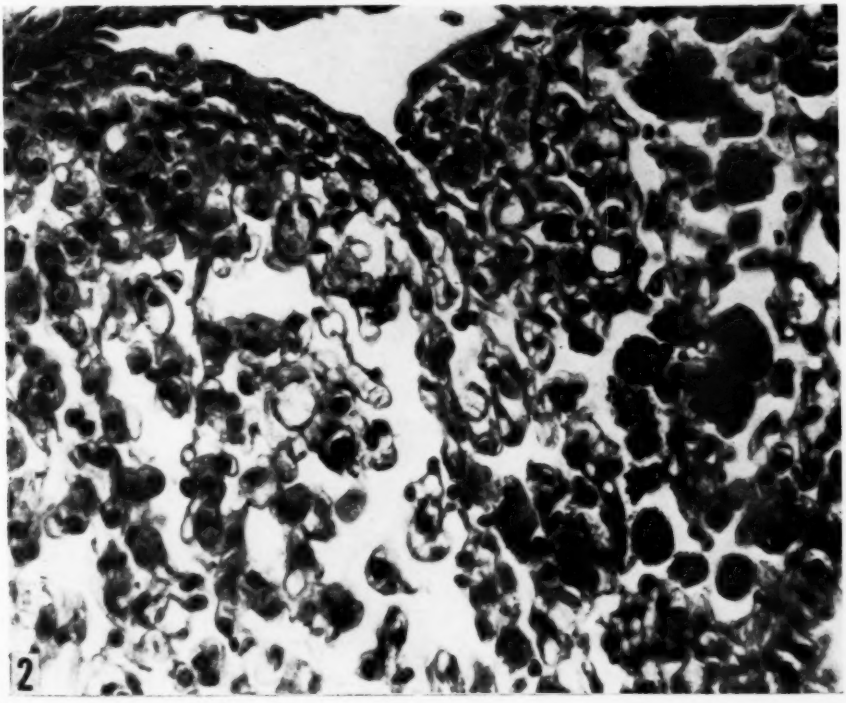


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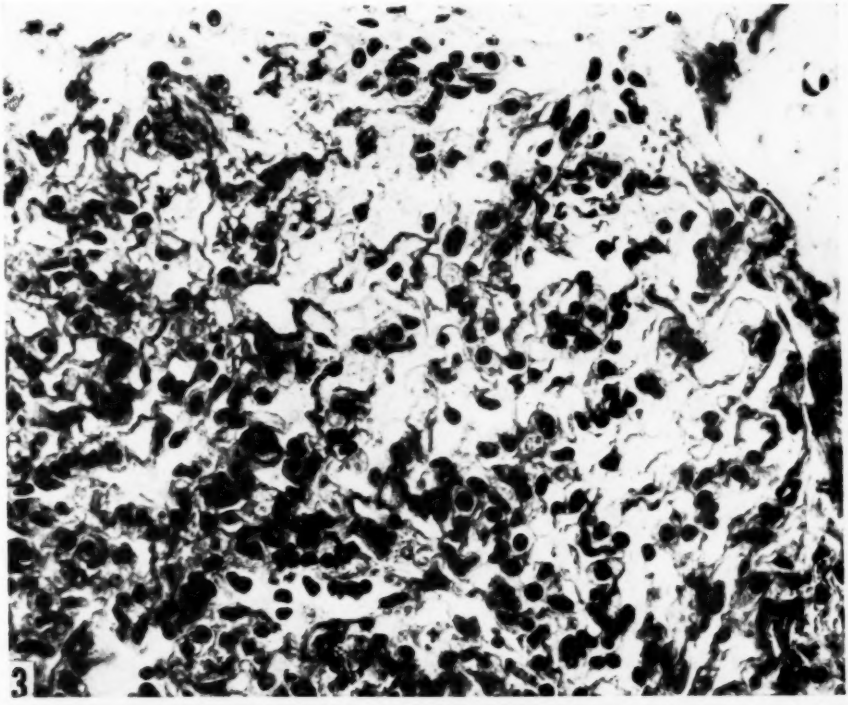
PLATE 32

Changes observed in the transplanted lung tissues:

FIGURE 3.—Focus of increased cellularity. The alveolar cells are larger and more prominent than usual, and some with large hyperchromatic nuclei are seen. Hematoxylin and eosin. × 500

FIGURE 4.—Development of squamous epithelium with some keratinization. Hematoxylin and eosin. × 500



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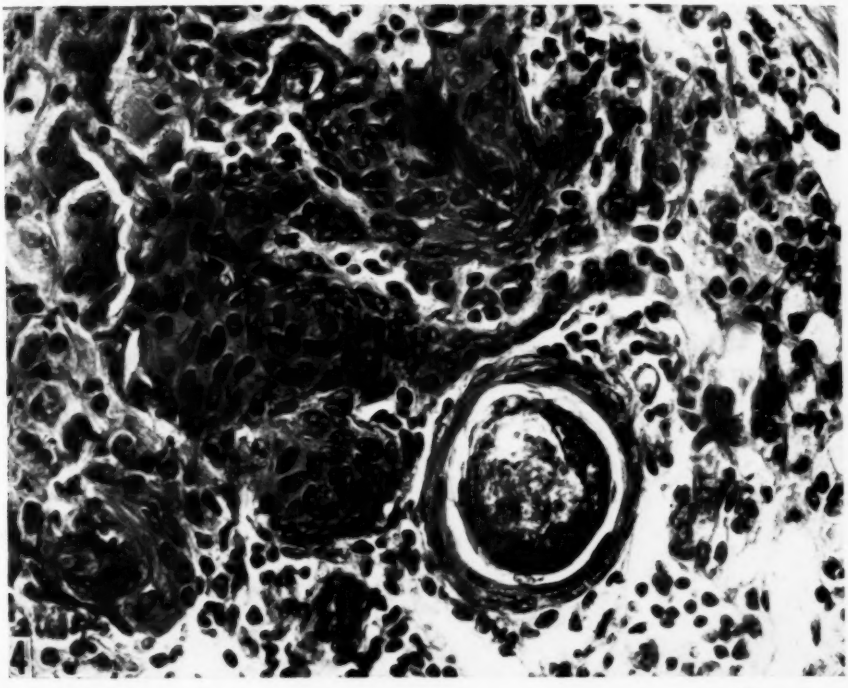

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Leukoedema and Keratosis in Relation to Leukoplakia of the Buccal Mucosa in Man[1]

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A generalized, whitish-gray lesion of the buccal mucosa has been frequently observed in asymptomatic patients of the Outpatient Department of Vanderbilt University Hospital. Biopsy specimens of the mucosa revealed intracellular edema and hyperplasia of the epithelium similar to lesions described by other investigators (*1–3*) as characteristic of leukoplakia. However, some of the generally accepted clinical features of leukoplakia were absent, leading us to believe that this condition and leukoplakia, although related, are not identical. We have called this condition *leukoedema*. Abnormalities of the oral mucosa that resemble this condition have been described in nutritional surveys under such terms as asymptomatic stomatitis or as deficiency stomatitis, and in papers dealing with oral leukoplakia under the terms incipient leukoplakia plana, pseudoleukokeratosis, and "filmy" leukoplakia.

This paper deals chiefly with the clinical and histologic features and the etiology of leukoedema and keratotic lesions of the buccal mucosa.

Materials and Methods

For this study a large group of white and Negro patients of both sexes, who would be available for frequent observation over a long period and would remain under the same stable environment in respect to diet and other factors, was desired. Such patients were found in the Central State

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Hospital for the Mentally Ill at Nashville, Tennessee. A survey was made of 646 ambulatory patients at this institution who were able to cooperate in permitting examination (tables 1, 2, 3). The patients were selected at random. Examination revealed that 56 percent of the patients had some degree of leukoedema of the buccal mucosa; fourteen percent were suffering with abnormalities of keratinization, and the majority of these also had leukoedema.

Recognition of leukoedema is facilitated by a good light source. Examinations of the oral cavity were made in direct sunlight or with a daylight "spot" lamp and head mirror. Abnormalities were confirmed by two or more observers. The hospital records of those patients with abnormalities of the buccal mucosa were reviewed with respect to age, hospitalization, and serology. The tobacco habits of each patient were described by the ward attendant. Routine examination was made of the conjunctiva, and in some patients of the larynx, vagina, and rectum, to determine whether the leukoedema may have been a generalized condition involving other epithelial structures. The patients with leukoedema or leukoplakia were later re-examined. A line sketch and a colored photograph were made of the buccal mucosa, lips and tongue to show the area and extent of involvement.

Biopsies of the buccal mucosa were taken of 105 patients. Most of these had leukoedema. Biopsies of the larynx were made in 2 cases and of the posterior wall of the vagina in 13. Xylocaine [6] (Lidocaine hydrochloride) as a 5-percent ointment, applied topically for approximately 90 seconds, was used as an anesthetic in securing biopsy specimens. This procedure minimizes distortion of the tissue that occurs when a fluid is injected. A suture on an atraumatic needle was used to fix and elevate the epithelium so that a section 5 × 10 mm. in size could be excised with a scalpel. The tissue was placed surface down on a small piece of paper as an aid in obtaining a true cross section. The tissues were fixed in 10 percent formalin, except in a few instances in which a solution of alcohol-formalin [7] was used. The defect was closed with a fine catgut suture. All biopsy sites healed within 3 days. Histologic sections were prepared with hematoxylin and eosin. Frozen sections were made of a few selected specimens and examined unstained, or stained with hematoxylin and eosin, toluidine blue, or Unna's picronigrosin. Measurements for epithelial thickness and cell diameter were made of hematoxylin-eosin preparations.

The hydrogen ion concentration of the saliva was determined with the Beckman *p*H meter. Saliva was obtained from 18 Negro men with leukoedema approximately 4 hours after the noon meal. The mouth was rinsed with warm tap water and the saliva collected in two portions of about 5 cc. each. The first of these was discarded and the second used for the analysis. Cultures and smears were made of the gums of 13

[6] The Xylocaine ointment was supplied by Astra Pharmaceutical Products, Inc., Worcester, Mass.
[7] Equal parts of 10 percent formalin and 95 percent alcohol.

Negro and 16 white men with leukoedema. The specimen was taken from the gingival margin after the removal of debris.

Observations

Histology of Normal Buccal Mucosa of Man

In the adult, the normal buccal mucosa (fig. 9) appeared pink, thin and smooth, with a readily perceptible vascular network. In the Negro, the mucosa was darker than in the white race. The pigmentation occurred diffusely or in maculae of varying size and shape. The mucosa could be lifted easily into folds, being loosely attached to the submucosa. Safranine or gentian violet disappeared within 4 hours after application on the surface.

Sections of a biopsy (fig. 1) showed that the surface of the epithelium was smooth. In the adult, the epithelium was of uniform thickness, measuring 200 to 400 microns, and the cell counts ranged from 14 to 17 from the tip of the rete peg to the surface (*4*). There was no evidence of keratinization. The outer zone was composed of stratified squamous cells, the cell outlines of which were difficult to ascertain although their nuclei were intact. In hematoxylin and eosin preparations, this outer layer was faintly acidophilic. There was no true granular cell layer; however, deeply stained large granules were irregularly dispersed in the intermediate portion of the squamous-cell zone. Intercellular bridges in this outer zone could not be identified. Beneath the squamous-cell zone, the cells were faintly basophilic and diamond-shaped with disorganized cytoplasm and elongated nuclei. In the remainder of the Malpighian layer, the cells were moderately basophilic and polygonal in shape; the intercellular bridges and cell contents (cytoplasm and nuclei) appeared normal. The basal layer consisted of deeply basophilic cuboidal or columnar cells with an occasional "clear cell." Nucleoli were distinct. Cells in mitosis were seen in about every third high-power field in the basal and adjacent layers. The basement membrane was not discernible in hematoxylin and eosin preparations. The lamina propria measured about 200 microns in thickness. The papillae were narrow and frequently almost reached the surface.

According to Jordan (*5*), mixed salivary glands are present in the buccal mucosa, and sebaceous glands are found at the margins of lips but occur rarely in the neighboring portions of the buccal mucosa. In the present material, no salivary glands were found in any of the biopsies of buccal mucosa. However sebaceous glands have been seen in all portions of the buccal mucosa as well as in the region adjacent to the lips. These glands were especially prominent along the lines formed by the fusion of the components of the face in embryonic development. These are the so-called "Fordyce spots" (*6*), which are yellow and measure up to 1 mm. in diameter.

Histopathology of Leukoedematous Lesions

Grossly, the mucosa appeared edematous. The degree of involvement ranged from a filmy opalescence to a whitish-gray or "waterlogged" appearance. In the advanced stages, the surface was coarsely wrinkled (fig. 10). The vascular network and the smooth or fine-grained appearance of the normal mucosa were obliterated.

The condition usually occurred bilaterally, frequently involving the entire buccal mucosa, including the oral surface of the lips. It was most noticeable along the occlusal line and in the middle and posterior areas of the cheek. Impressions of the teeth along the occlusal line were frequently prominent in the more advanced stages of leukoedema. Ampulla of Stenson's duct was in most cases swollen. Desquamation may occur, leaving a "moth-eaten," eroded surface, especially opposite sharp teeth or poorly fitting dentures (fig. 11). These areas remained asymptomatic without bleeding and did not appear inflamed. Occasionally, leukoedema occurred as a localized patch (fig. 12), which may be indistinguishable from leukoplakia without a biopsy. In severe stages of leukoedema and in areas of erosion, safranine and gentian violet dyes were retained for periods of 18 to 24 hours.

The four histologic features of leukoedema were (figs. 2, 3, 15):

1) Increase in thickness of the epithelium. In severe degrees, the overall thickness may measure 900 to 1,000 or more microns. In moderate degrees of leukoedema the epithelium was commonly 600 microns thick, and in slight degrees of leukoedema it measured 300 to 500 microns. In addition to intracellular edema, some of the increased thickness was evidently due to hyperplasia. In the moderate and severe degrees of the disease, the number of epithelial cells increased by 50 to 100 percent over the normal. Counts in the moderate and severe degrees ranged from 24 to 36 cells per cross section.

2) Intracellular edema of the Malpighian layer. The cells were very large and pale, measuring from 20 to 50 microns in diameter and staining slightly basophilic. The polygonal shape of the cells was maintained and frequently extended to the surface without becoming flattened. A lattice or netlike arrangement resembling areolar tissue may be present near the surface. In these latter areas, the cells appeared to have lost their cytoplasm, although on closer study the cytoplasm was evident by a faint ground-glass appearance. Single cells or groups of cells, larger and paler than the surrounding cells, were frequently seen. The origin of these was unknown. Intercellular bridges were indistinct throughout but the cellular outlines were fairly well retained. The nuclear material was often disintegrated and concentrated toward one side, with the remainder of the nucleus clear. In contrast to the normal, the nucleolus was only occasionally seen.

3) An irregular surface without keratinization or increase in "granules." In some cases there was a layer, up to 200 microns thick, of faintly acidophilic squamous cells with indistinct cell outlines but with clearly discernible, though flattened, nuclei. This surface layer was amorphous,

and might be partially detached from the underlying Malpighian layer during sectioning.

4) Broad rete pegs, frequently elongated and usually irregular in depth.

The basal cell layer in leukoedema appeared to be normal with respect to mitosis and arrangement of cells. There was no consistent abnormality of the lamina propria. In some cases, a slight to moderate increase in lymphoid cells was present.

Angular stomatitis and buccal erythema were seen in a few patients of this series; apparently neither was part of the syndrome. They were not observed in the most severe cases of leukoedema and may disappear without any accompanying change in the leukoedema.

For the purposes of this study, the degree of leukoedema has been arbitrarily assessed as slight, moderate, or severe. These conditions are defined as: *Slight*—mucosa was opalescent, thickened, and with the capillary network obliterated (fig. 12); the entire mucosa may be involved but the middle and posterior cheek were principally affected; the edematous appearance disappeared upon stretching. *Moderate*—mucosa possessed a coarsely granular surface (fig. 11) with a definite white or whitish-gray appearance that persisted upon stretching. *Severe*—mucosa appeared grayish white and thickened (fig. 10); in some cases it appeared shaggy and wet, and in other instances, leathery and dry.

Abnormal Keratinization

Leukoplakia.—For this discussion the lesion of leukoplakia may be defined clinically as a white "patch" of epithelium with a well-defined border of varying size and shape that cannot be removed by scraping. In some cases, multiple areas of involvement were found. The surface may or may not be elevated above the surrounding mucosa, and it may be smooth or finely wrinkled or verrucose in nature (fig. 13). Histologically (figs. 4, 5) the lesion was characterized by a definite keratinized outer or surface layer with practically no detectable nuclei, a granular cell layer, and distinct intercellular bridges in the Malpighian layer. Mitotic activity was not a prominent feature. There was usually an increase in lymphoid cells in the lamina propria.

Keratosis.—Areas of keratosis (figs. 14, 15), not typically leukoplakia, were observed at sites exposed to drying and trauma. Most frequently, these areas appeared as wedges of epithelium with either the apex or the base of the wedge at the anterior commissure. When they occurred at this site they were usually bilateral. The epithelium in these areas appeared dry, crinkled, and thickened, and occasionally it was eroded. The areas varied in color from brownish pink to grayish white. Gray flecks were occasionally seen. Localized areas of keratosis were observed (fig. 13) in the form of an outpocketing at the site where the tobacco quid is habitually held. They usually occurred unilaterally in the inferior alveolar sulcus and measured up to 3 or 4 cm. in diameter. The surface appeared dry, crinkled, erythematous, and brown in color.

Microscopically, (figs. 6, 7) the lesion revealed the features of para-

keratosis, that is, retention of nuclei in the keratinized surface layer and a distinct granular layer. The surface was slightly irregular, and the stratified keratinized layer measured about 25 microns in thickness. Many of the nuclei had been retained, but they were flattened. A layer of granular cells was found either adjacent to this keratinized layer or deeper in the epithelium, but this layer was not as striking as it is in true leukoplakia. The cells of the granular zone were flattened or swollen with indistinct cellular outlines, their nuclei being retained. In some cases there was a network of vacuolated cells superficial to the granular zone. The cells underlying the granular zone were swollen and their cellular outlines indistinct. Intercellular bridges were or were not seen depending upon the extent of edema. This intracellular swelling extended down into the rete peg. The basal layer was one to two cells thick. The cellular outlines and the nuclei of the basal cells were distinct. The rete pegs might be broad and as irregular as in leukoedema. There appeared to be a slight increase of lymphoid cells in the lamina propria.

Leukoedema and Abnormal Keratinization

Leukoedema of the buccal mucosa was observed in 374 (58 percent) of the 646 patients examined (table 1). It occurred in 43 percent of the white and 90 percent of the Negro patients. The susceptibility of the Negro to this condition is further supported by the fact that a larger percentage of Negroes develop the more severe stages of leukoedema than white persons. Among both Negroes and whites, the incidence was slightly higher in men than in women, while the percentage with the severe stages was considerably higher in men.

TABLE 1.—*Prevalence of leukoedema of buccal mucosa, Central State Hospital, initial examination, August 1951*

	Total	Degree of leukoedema				Total positive	
		None	Slight	Moderate	Severe	Number	Percent
White male	209	115	58	25	11	94	45
White female	228	136	74	16	2	92	40
Negro male	109	7	23	33	46	102	94
Negro female	100	14	32	27	27	86	86
Total	646	272	187	101	86	374	58

Histologically, keratosis appeared to have some features in common with both leukoplakia and leukoedema in that intracellular edema, a granular cell layer, and a slight keratinization were present.

Table 2 shows the frequency of occurrence of abnormalities of keratinization of the buccal mucosa. Eighty-nine, or about 14 percent, of the patients examined had localized areas of abnormal keratinization. In 17 of the cases there was more than one type of keratosis. Abnormal keratinization occurred least often in white women than in the other patients, and it was most prevalent in Negro men. Leukoplakia occurred in 3.4 percent of the 646 patients, being significantly higher in the Negro than in the

white patients. There was no sex difference in the occurrence of leukoplakia. Clinical examination revealed that 6.8 percent of all patients examined had localized whitish areas resembling leukoplakia; however, microscopic study of biopsy sections showed half of such lesions to be those of leukoedema. Dry, crinkled areas of keratosis occurred in 6.7 percent of the entire group of patients, but were confined principally to the men. The "tobacco pouch" type of keratosis occurred in 19 patients. Seven of these 19 had leukoplakia elsewhere on the buccal mucosa. Seven men, not included in the table, had leukoplakia of the external lip.

TABLE 2.—*Prevalence of abnormalities of keratinization of the buccal mucosa, Central State Hospital, August 1951*

Group	Cases examined	Leukoplakia		Localized* leukoedema		Dry crinkling		"Tobacco pouch"		No. of cases with one or more lesions	
		Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
White male	209	3	1.4	12	5.7	27	12.9	5	2.4	42	20.1
White female	228	5	2.2	4	1.8	1	0.4	5	2.2	12	5.3
Negro male	109	9	8.3	5	4.6	11	10.1	5	4.6	22	20.2
Negro female	100	5	5.0	1	1.0	4	4.0	4	4.0	13	13.0
Total	646	22	3.4	22	3.4	43	6.7	19	2.9	89	13.8

*Gross appearance compatible with leukoplakia; microscopically shows only intracellular edema and hyperplasia.

Table 3 shows the association of abnormal keratinization and leukoedema of the buccal mucosa. The seven patients with leukoplakia of the external lip also had leukoedema. There was no relation between the severity of the leukoedema and the incidence of leukoplakia in any of the patients. The few cases of abnormal keratinization in the advanced stages of leukoedema may possibly result from the failure to recognize features that were masked in the edematous mucosa of leukoedema. Keratinization without leukoedema occurred in only one case.

TABLE 3.—*Association of abnormalities of keratinization to degree of leukoedema, Central State Hospital, initial examination, August 1951*

	Total	Degree of leukoedema			
		None	Slight	Moderate	Severe
No keratinization	579	271	155	80	73
Localized leukoedema	22	0	11	8	3
Abnormalities of keratinization:					
Leukoplakia	22	0	11	6	5
Keratoses:					
Dry crinkling	43	1	23	12	7
"Tobacco pouch"	19	0	8	5	6
Total cases with keratinization*	67	1	32	21	13
Total cases	646	272	187	101	86
Percent with keratinization	.....	.....	17.1	20.8	15.1

*Some patients had more than one type of lesion; for this reason, the total number of cases with keratinization is less than the sum of the values listed in that column.

Etiologic Factors of Abnormal Keratinization and Leukoedema

Age and time of residence in hospital.—The average age of the patients examined was 45.6 years, the range being 15 to 70 years. The average time of residence in the hospital was 7.0 years. The factors of age and length of residence were found to be unrelated to the incidence of either keratosis or leukoedema.

Blood serum.—Syphilis did not appear to be a factor in the etiology of these disorders. Fourteen of the 188 Negro patients with leukoedema had a diagnosis of syphilis on admission, and all were adequately treated. None of the 186 white patients with leukoedema had a positive test for syphilis on admission.

Tobacco habits.—Eighty percent of the men (including white and Negro) and 25 percent of the white women used tobacco. Usually this was either snuff or chewing tobacco, although most white men smoked cigarettes or pipes. It was not possible to obtain accurate information on the tobacco habits of the Negro women. Table 4 shows that the non-users of tobacco were less prone to develop keratinization than those who used tobacco. In order to establish fully the injurious effect of tobacco on the mucosa, the extent and length of previous use and the form of the tobacco would need to be evaluated. No relationship was found between tobacco habits and incidence of leukoedema.

TABLE 4.—*Relation of tobacco to abnormalities of keratinization of the buccal mucosa (selected groups)*

	White female				White male				Negro male			
	Keratinization				Keratinization				Keratinization			
	Total	None	Positive		Total	None	Positive		Total	None	Positive	
			Number	Percent			Number	Percent			Number	Percent
Tobacco	56	52	4	7	47	19	28	60	45	31	14	31
No tobacco	172	166	6	3	13	12	1	8	10	10	0	0
Total	228	218	10	4	60	31	29	48	55	41	14	25

Oral hygiene.—Although poor oral hygiene was prevalent except in about 10 percent of the patients who were edentulous, neither bacterial infection, the *p*H of the saliva, or galvanochemical stimulation appeared to be related to the development of either leukoedema or keratosis. Bacteriological examination of material from the gingival margin of 29 male patients showed the following pathogenic organisms: *Streptococcus hemolyticus* in 10 patients, *Str. viridans* in 16, mixed culture of the above in 1, and mixed *Str. viridans* and *Staphylococcus aureus* in 2 patients. Smears failed to show *Borrelia vincenti* as was expected from those with ulcerative gingivitis. The hydrogen ion concentration of the saliva from the 18 Negro men ranged from *p*H 6.3 to 7.4, which is within normal

limits. Since dental fillings were infrequent, it is unlikely that galvanochemical stimulation was an etiologic factor in either leukoedema or keratinization. Chronic irritation from sharp or irregular teeth appeared to be a prominent factor in the development of keratoses since the mucosa opposite such teeth was frequently keratotic.

Larynx

Satisfactory mirror laryngoscopy[8] was accomplished in 27 of 47 Negro men who had some degree of buccal leukoedema. Anesthesia was not used. The glottis was abnormal in all of the 27 patients. The mucosa was opalescent or erythematous in 18 patients, and the posterior third of the true vocal cords of 18 of the patients was highly vascular; the mucosa was thickened in the posterior commissure of 21, and the false vocal cords were hypertrophied in 2 patients. In several, the hypertrophied mucosa in the interarytenoid area formed a distinct mass, was opalescent in appearance, and interfered with approximation of the cords. This may represent a variety of hypertrophic laryngitis (*7*). A biopsy was performed on one subject with the interarytenoid mass. Histologigically, the sections showed marked intracellular edema and hyperplasia of the epithelium (fig. 8).

Rectum

Proctoscopic examination of the rectal mucosa for a distance of 7 to 8 cm. above the anus of 6 white and 7 Negro male patients who had some degree of buccal leukoedema showed no gross evidence of leukoedema.

Conjunctiva

Examination of the palpebral and bulbar conjunctiva under daylight failed to reveal any abnormality resembling the epithelial changes seen in the oral cavity.

Vagina and Vulva

The surface of the vagina and vulva was examined without reference to the menstrual cycle. A number of the Negro and white women in the pre- and postmenstrual periods showed epithelial changes in the vulva and vagina that resembled the lesions of leukoedema of the buccal mucosa. Biopsy sections taken from the mid-third of the posterior wall of the vagina showed intracellular edema and hyperplasia. This represents simply a preliminary observation, since the epithelium of some of the tissues resembled that of the follicular or the luteal phase of the menstrual cycle (*8*).

Discussion

Leukoedema of the buccal mucosa is of interest because of its frequency and its probable relation to leukoplakia. Similar clinical disorders such

[8] We wish to thank Dr. J. Thomas Bryan, Attending Otolaryngologist, Vanderbilt University Hospital, for aid in making these examinations.

as asymptomatic stomatitis (*9*), pseudoleukokeratosis (*10*), incipient leukoplakia plana (*11*), and "filmy" leukoplakia have been observed during nutrition surveys in the United States (*9*), in intern camps in Singapore (*12*), in the civilian population in the Netherlands (*13*), and in the Greenland Eskimo (*11*). The opalescent to opaque, white to grayish appearance was mentioned in a discussion of the transitional phases of "leukoplastic" conditions of the oral mucosa (*14*). It is probable that when leukoedema has been observed in the buccal mucosa in its advanced stage, either as local or widespread lesions, the condition has been diagnosed as leukoplakia. A detailed histologic description of leukoedema has been presented because of the pathologic implications of leukoplakia, which it closely resembles. Indeed, certain texts of pathology (*1–3*) give illustrations of leukoplakia that show the intracellular edema and hyperplasia of leukoedema without the keratinization characteristic of leukoplakia.

The present studies, as well as those referred to, suggest that leukoedema and keratotic deviations (*15*) of the buccal mucosa and of other sites (*16*) are more common than is generally recognized. This may be accounted for by the fact that both are asymptomatic except in the advanced stages, and are not readily observed without the aid of a good light source.

The etiology of leukoedema is not known. Its occurrence in transitional epithelium in several sites suggests a metabolic or a nutritional defect rather than a reaction to local irritants. It has been generally recognized since the early description of leukoplakia by Virchow that a number of local irritants, such as tobacco and long-continued trauma from sharp teeth, are etiologic factors in the development of leukoplakia (*17*). This was corroborated in the present series of cases.

Leukoedema may represent a lesion of the mucosa in which leukoplakia and other keratoses are more likely to develop than in normal epithelium. This is supported by the findings that: 1) all but one of the patients having either keratosis or leukoplakia had some degree of leukoedema in the adjacent mucosa; 2) the sections showing parakeratosis, a condition frequently found in leukoedematous mucosa of the cheek but not a part of that entity, have features of both leukoplakia and leukoedema, *i.e.* an amorphous keratinizing superficial layer and granular cell zone of the former and intracellular edema of the latter; and 3) chronic irritation from tobacco and the trauma from biting are more likely to result in keratosis or leukoplakia in the leukoedematous buccal mucosa than in the normal mucosa.

Summary

The histology of biopsy specimens of 105 patients revealed that most of them suffered from a disorder termed "leukoedema" of the buccal mucosa. This is an asymptomatic disease of stratified epithelium, the etiology of which is unknown. It is suggested that leukoedema may represent a lesion of mucosa in which leukoplakia or other keratoses are more likely to develop than in normal epithelium. Leukoedema occurs not

only as diffuse or localized areas, but also around "patches" of leukoplakia with which it may merge.

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PLATE 33

FIGURE 1.—Normal buccal mucosa. The biopsy was sectioned slightly obliquely. The surface is smooth. There is no keratinization or granular cell layer and no intracellular edema. Case H. R. S.: White male, age 47 years. Hematoxylin and eosin. × 80

FIGURE 2.—Lesion of moderate leukoedema of the buccal mucosa. Biopsy from the eroded area in the right anterior buccal mucosa. Surface irregular; sloughing of nonkeratinized outer layer. Remainder of Malpighian layer shows intracellular edema. Rete pegs irregular. Case 47: Negro male, age 42 years. Hematoxylin and eosin. × 80

FIGURE 3.—Localized white "patch" of slight to moderate leukoedema of the right posterior buccal mucosa. Intracellular edema and other features similar to Case 124 (figs. 8 and 10). Case 236: White male, age 32 years. Hematoxylin and eosin. × 80

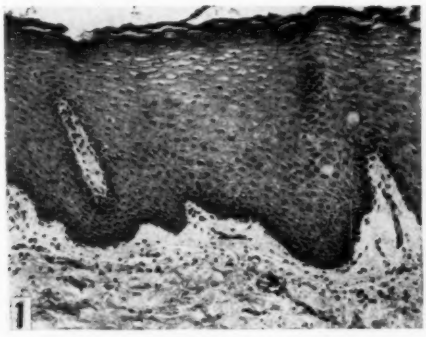
FIGURE 4.—Photomicrograph of keratotic area in the buccal mucosa. Biopsy from the posterior inferior sulcus as shown in figure 13. This illustration shows features of extreme hyperkeratosis with abrupt transition to a parakeratotic area which in turn changes to an area of leukoedema. The thickness of the granular cell layer is in direct proportion to the density of the keratin layer. The tunica propria shows slight increase in chronic inflammatory cells and a moderate increase in vascularity. Case 119: Negro male, age 40 years. × 10

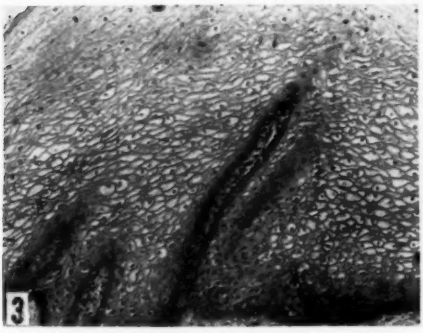
FIGURE 5.—Photomicrograph of a leukoplakia in the buccal mucosa. Biopsy from the verrucose lesion shown in figure 13. There is a dense layer of keratin overlying a prominent granular cell layer. The tunica propria shows a slight increase in chronic inflammatory cells and a moderate increase in vascularity. Case 119: Negro male, age 40 years. Hematoxylin and eosin. × 80

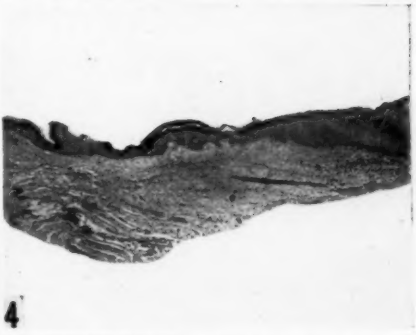
FIGURE 6.—Photomicrograph of slight to moderate leukoedema. Biopsy 2 cm. posterior to anterior commissure. Surface covered by thin layer of keratin overlying a thicker parakeratotic layer which in turn overlies a broad granular layer. The Malpighian layer is not remarkable except for slight thickening and an irregularity of rete pegs. The tunica propria shows an unusual number of inflammatory cells. Case 7: Negro male, age 51 years. Hematoxylin and eosin. × 80

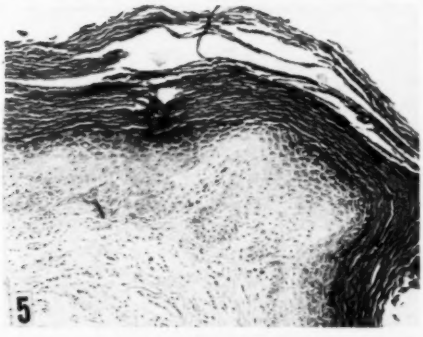
FIGURE 7.—Photomicrograph of keratotic area. Biopsy taken from right anterior buccal mucosa at site of dry, crinkled area. The surface is covered by a moderately dense layer of keratin; underlying it is a narrow zone of granular cells. There is no intracellular edema and no increased thickness of the Malpighian layer. Case 25: Negro male, age 46 years. Hematoxylin and eosin. × 80

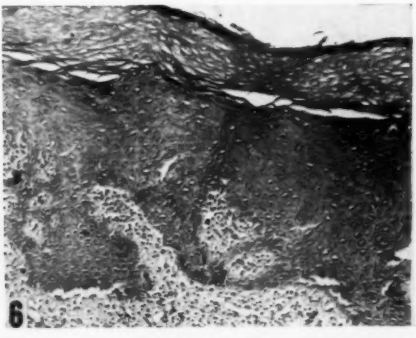
FIGURE 8.—Biopsy of the posterior commissure of the larynx. Note resemblance of the epithelial hypertrophy and intracellular edema to that shown in figures 1, 10, 11. Case 124: Negro male, age 52 years. Hematoxylin and eosin. × 80











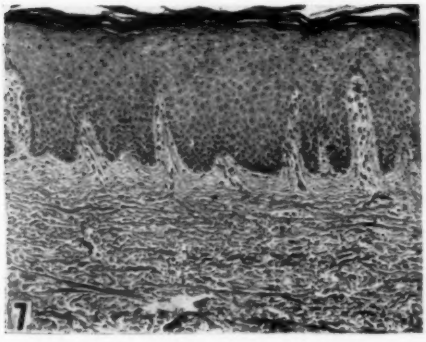


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PLATE 34

FIGURE 9.—Normal buccal mucosa. The mucosa is pink in color, and there is an absence of opalescent keratinization. Case H. S.: White male, age 20 years.

FIGURE 10.—Generalized severe leukoedema. Case 124: Negro male, age 52 years.

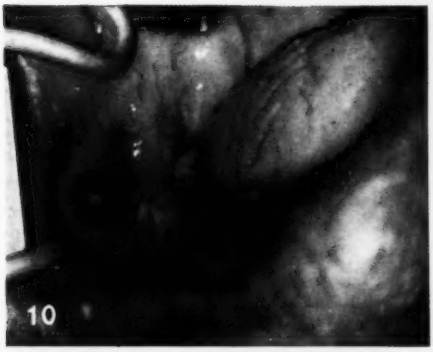
FIGURE 11.—Moderate leukoedema of the buccal mucosa. Irregular wedge-shaped whitish patch in mid-cheek with erosion at anterior commissure, extending to the oral surface of the lips. Asymptomatic and bilateral. Case 47: Negro male, age 42 years.

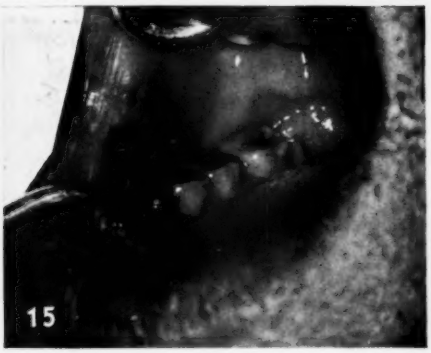
FIGURE 12.—Slight to moderate leukoedema. Note white area in posterior buccal mucosa (opposite third molar) that resembles leukoplakia. Case 236: White male, age 32 years.

FIGURE 13.—Verrucose hyperkeratotic area (leukoplakia) at the site of a "tobacco pouch" (in sulcus opposite second molar). Surrounding epithelium shows moderate leukoedema. A similar, nonverrucose lesion is present in the opposite cheek. Case 119: Negro male, age 40 years.

FIGURE 14.—Slight to moderate leukoedema. Extensive bilateral dense-appearing white fissured keratotic area resembling leukoplakia, occupying anterior half of buccal mucosa and extending to the vermillion border of the lips. Case 7: Negro male, age 51 years.

FIGURE 15.—A dry, crinkled, wedge-shaped keratotic area appears at the anterior commissure (bilateral). The remainder of the buccal mucosa shows slight leukoedema. Case 25: Negro male, age 25 years.





Sandstead and Lowe

The Concentration of Myelokentric and Lymphokentric Acids from Serum and Plasma of Leukemic Patients [1, 2]

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In earlier work (*1–3*) it was found that the urine of patients with leukemia contained two substances, myelokentric acid and lymphokentric acid, which produced characteristic cellular changes when injected into guinea pigs. Foster and Miller (*4*) investigated the biological effect of injecting guinea pigs with small quantities of serum from patients with leukemia. This biological method differs considerably from that employed in the work on urine. The test lasts only 24 hours and requires only a single dose, and therefore has great advantages in speed and convenience as compared with the methods previously used. The biological effects, however, are not as marked. The reactions are most apparent in the lymph nodes, and the injection of serum of patients with each type of leukemia—myeloid, lymphoid, or monocytic—produces a characteristic reaction for each type.

In the present investigation, it was found that extracts of the leukemic serum or plasma produce the same effects described by Foster and Miller (*4*). It was possible to concentrate the chemical agents involved so that the reactions can be produced in quantities of 20 μg. or less. It has not been possible to isolate pure substances, because of the small amount of leukemic blood available.

Experimental Observations

Biological methods.—Activity was detected by the test of Foster and Miller (*4*). The test substances were injected intraperitoneally into guinea pigs in the form of a solution of the sodium salt in a 0.5 M phosphate buffer at *p*H 7. A constant volume of 5 ml. was used throughout. The buffered solution was freshly prepared for each test to avoid bacterial contamination. Esters were hydrolyzed to the free acid by adding to the test sample 5 ml. of ethanol and 0.1 ml. of 45 percent potassium hydroxide [4] and refluxing for 15 minutes on the steam bath. The ethanol was then

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[3] Miss Ruth M. Horcher gave valuable technical assistance. We wish to thank Mr. M. R. Lipkin, Dr. E. J. Rosenbaum, and Dr. Abraham Schneider of the Sun Oil Company for their help.
[4] The term "percent" means grams per 100 grams of the solution in water.

removed *in vacuo* on the steam bath and the residue was adjusted to *p*H 7 with dilute sodium acid phosphate solution. At least two guinea pigs were used for each test. The histologic results are designated according to type by M, L, and H, for lesions of the myeloid, lymphoid, and "mixed" type, respectively (table 1). These reactions in the lymph nodes of the guinea pigs are the same as those described by Foster and Miller (*4*). The myeloid reaction consists of lymphocytic destruction, depletion of the number of lymphocytes, edema of the intercellular spaces, pleomorphism of the cellular structure, a slight infiltration of polymorphonuclear leukocytes, a loss of follicles, and an apparent increase in the underlying connective tissue. There are a few scattered eosinophils. The lymphoid reaction consists of an increase in the size and number of lymphoid follicles, as well as an increase in the lymphocytic collars around these follicles; although in some strongly positive (++) animals, a dense population of the lymphoid cells of uniform size is found. The nodes are frequently compact and are usually densely populated with cells. There is very little intercellular edema. The mixed (H) reaction usually shows an increase in lymphopoiesis and some pleomorphism with a few polymorphonuclear leukocytes. Often there is an increase in fibroblasts and eosinophils.

For each of the three types of reaction, differences of degree are designated by "slight," + and ++. The slight reaction is possibly equivocal, the + reaction is definitely positive, while the ++ reaction shows much more than the others.

The doses are given in terms of serum or plasma volume; this calculation is based on the assumption that the fraction being tested represents the volume of plasma or serum from which it was derived. This assumption will be true when the separation is complete, but not when the activity is distributed through the fractions separated.

Extraction Procedure

(*a*) *Acetone precipitation followed by extraction with chloroform.*—The serum used was fresh and contained no additives. The plasma contained an anticoagulant (glucose, sodium citrate and citric acid) and had a *p*H of about 6.5.

The serum (or plasma) was mixed with 3.5 volumes of acetone, and the mixture was allowed to stand overnight at room temperature. The precipitated protein was separated and stirred into a paste with 0.12 M phosphate buffer, *p*H 7.0 (½ of the volume of the original plasma) using a mechanical stirrer. The resulting paste was allowed to stand overnight; it was then treated with 3.5 volumes of acetone (calculated with reference to the volume of buffer). The protein residue was separated as before and discarded.

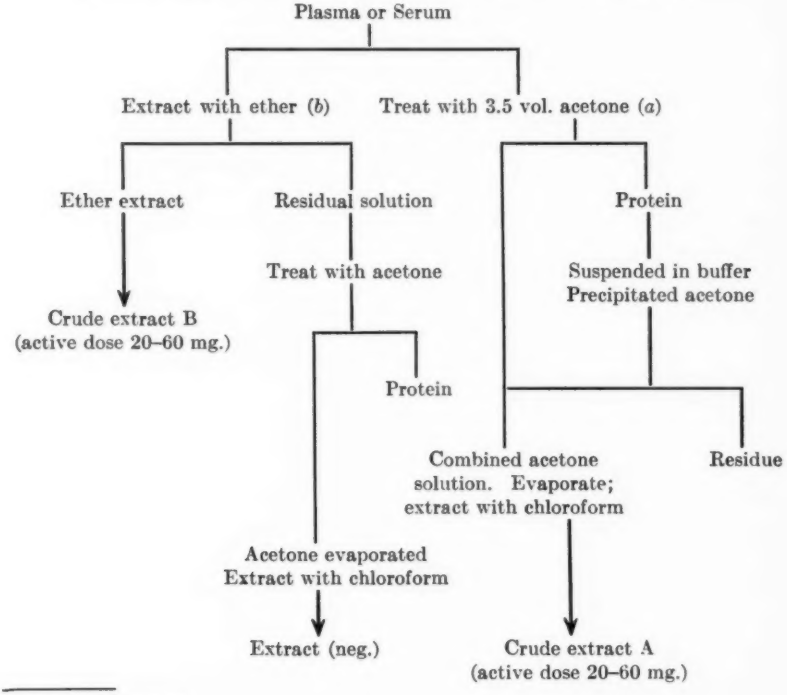
The acetone solutions from the two precipitations were separately evaporated *in vacuo* on the steam bath to remove acetone completely.[5] The residual aqueous solutions were then cooled, and extracted with half

[5] The volume of the residual aqueous solution was ⅓ of the volume of the original plasma or buffer solution. The solution can be evaporated to dryness without loss of activity, but this requires special apparatus in order to prevent loss by foaming.

their volume of chloroform in five extractions. The aqueous solution was then made acid to congo paper with 10 percent hydrochloric acid [4] and re-extracted with chloroform. The combined chloroform extracts were dried over anhydrous sodium sulfate, filtered, and evaporated *in vacuo* on the steam bath until the solvent was removed.[5]

The residue remaining after the removal of the chloroform was used for animal tests. The residue derived in this manner from the first acetone precipitation is designated A-1 in table 1. The material derived from the second acetone treatment of the protein dispersed in buffer (A-2) was sometimes slightly active. Because of this activity in the extract from the protein precipitate, the final chloroform extracts of both portions were combined for subsequent manipulations. (This combined material is designated fraction A; see flow-sheet 1.) The weights of material extracted by the procedure varied widely from patient to patient (30 to 240 mg. per 10 ml. of serum or plasma). In some experiments, plasma dried by the lyophil method was used. The dried material was dispersed in ten times its weight of water and treated by the same method.

FLOW-SHEET 1.—*Concentration of active agents from leukemic serum or plasma*



[4] See footnote 4, page 439.

[5] This method for drying the solution and removing the solvent is called the "usual method" below. In these distillations, the temperature of the solution does not exceed 80° C. and the distillation is stopped as soon as the solvent is removed. In drying chloroform solutions, it is convenient to remove the sodium sulfate solution that separates on the surface with the aid of a capillary tube attached to a suction flask.

TABLE 1.—*Tests on active products*

Source	No. of Samples*	Fraction	Dose† (*ml.*)	Dose (*mg.*)	Activity‡
1) Crude Extracts					
Myeloid:					
serum	2	A-1	5	25–28	+M(4)
serum	1	A-2	10	—§	slight M
plasma	1	B	6	12	+M
Lymphoid:					
serum	2	A-1	10	50–60	+L(4)
serum	1	A-2	10	—§	+L
serum	1	B	6	16	++L
Monocytic:					
plasma‖	1	A	10	25	++H
Normal:					
plasma	1	A	40	100	neg.
			75	190	+H
			90	335	+H
			170	430	++H
2) Acidic Fractions¶					
Myeloid A	2	F	5	—§	+M(4)
" B	2	F	5	0. 2–0. 6	neg. to +M(4)
	1	F	10	1. 3	+M
			17	2. 2	++M
	1	F ester	17	0. 56	++M
Lymphoid B	1	F	6	1. 5	++L
	1	F	20	1. 6	+L
			30	2. 4	++L
Monocytic A	1	D	10	4	+H
			20	8	++H
3) Carbinol Separation					
Myeloid, nonketone	1	Noncarbinol	14	0. 12	+M
Lymphoid, F ester	1	Hydroxy-acid	6	0. 16	+L
			22	0. 6	++L
Monocytic, chromatograph fraction	1	Hydroxy-acid	20	0. 04	++L
		Noncarbinol	20	0. 16	++M

4) Girard Separation					
Myeloid F ester	1	Nonketones**	8	0. 14	+M
	1	Heptane-soluble††	7	0. 3	slight M
		Heptane-insoluble	7	0. 06	+M
5) Solubility in n-Heptane					
Myeloid F ester	1	Insoluble	12	0. 4	+M
Noncarbinol fraction of nonketones as ester	1	Insoluble	14	0. 009	+M
			36	0. 023	++M
Lymphoid:					
Hydroxy ester	1	Insoluble	25	0. 3	+L
	1	Insoluble	20	0. 05	+L
Monocytic:					
Noncarbinol fraction of nonketones as ester	1	Insoluble	20	0. 015	+M

*This refers to the serum or plasma of individual patients, each prepared separately. The diagnosis was made by competent hematologists, using blood smears and bone marrow aspiration in every case.

†Serum or plasma.

‡Two guinea pigs, except where noted in parenthesis.

§Not weighed.

||This was plasma dried by the lyophil method and stored for 1 year under refrigeration.

¶The letters A and B refer to the crude extracts hydrolyzed.

**Usual Girard separation, except that the nonketones were washed with dilute hydrochloric acid.

††The Girard reaction product was diluted with water, and extracted with heptane, then with chloroform.

(*b*) *Extraction with ether.*—In an alternative method, used for larger quantities, the serum or plasma (which must not be alkaline) was extracted five times with equal volumes of peroxide-free ether, and the combined ethereal solution was treated in the usual manner [6] to give a residue, B. After extraction, the serum was then treated by the method of (*a*) to give a product C merely to determine whether the activity was effectively removed by ether (table 2).

TABLE 2.—*Tests on discarded fractions*

Type of leukemia	Fraction	Dose* (*ml.*)	Dose (*mg.*)	Number of separate preparations; activity, if not negative
Myeloid:				
Serum	Soluble part of protein	10	—	1
Plasma	C	12	55	1
Plasma, serum	G	10–12	10	3
Plasma, serum	H	12	15	3
Plasma	Carbinols	10	0. 1	1
Plasma	Ketones	7	0. 1	1
Plasma	Ketones	8	0. 17	Slight M
Plasma	Esters soluble in *n*-heptane.	12	0. 6	1
Lymphoid:				
Plasma, serum	C, G, H and protein residue.	Doses and activity similar to above.		
Plasma	Noncarbinol esters	53	9	1 neg. to slight M
Monocytic:				
Plasma	E	20	15	1

*Serum or plasma.

Separation of Active Fractions into Acidic and Neutral Material

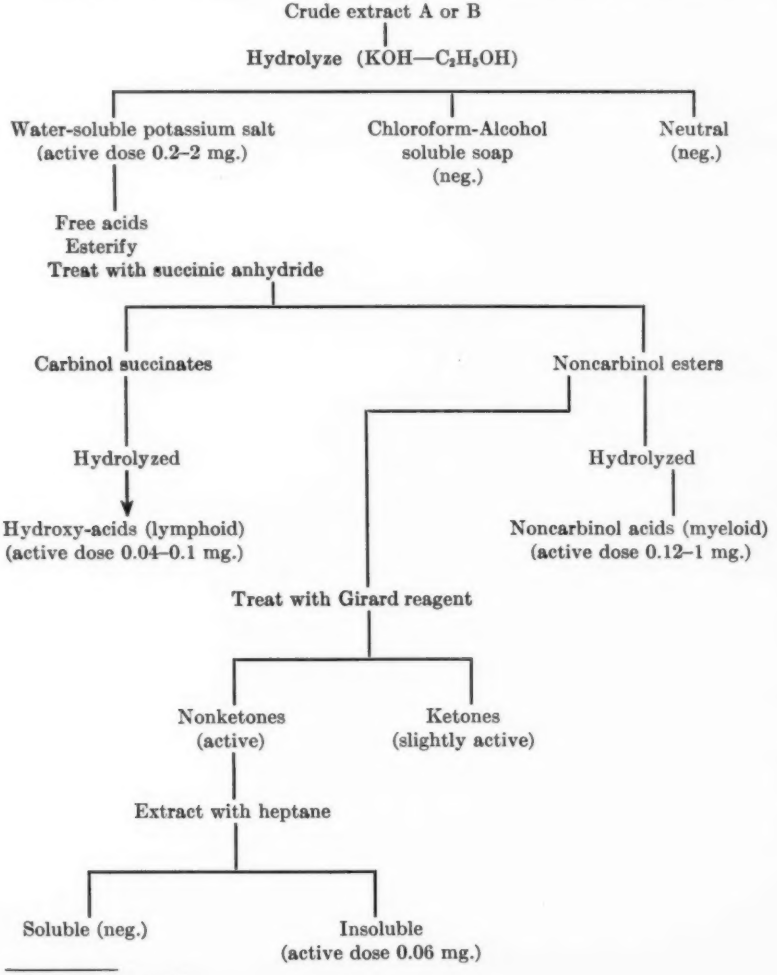
1) *By partition between alkali and chloroform-ether.*—A sample of fraction A from dried plasma in monocytic leukemia (0.5 gm.) was dissolved in 20 ml. of chloroform and extracted with 3 portions of 10 ml. of 0.5 N sodium hydroxide solution; the alkaline layer and emulsion was then extracted with 15 ml. of ether in order to break the emulsion. The separated aqueous layer was acidified with 10 percent hydrochloric acid [4] to congo paper blue, and then the precipitated material was taken up in fresh chloroform. The chloroform extract of the acidified aqueous layer (acidic fraction) was treated in the usual manner,[6] giving active material D. The chloroform originally used in the partition with alkali was combined with the ether extract of the alkali. This is the "neutral" fraction. Inactive neutral material, E, was obtained from this solution by the usual method.[6]

2) *By hydrolysis in hot alcoholic potassium hydroxide.*—Extracts of type A or B were refluxed in a solution containing 5.7 ml. of ethanol and 2.3 ml. of 45 percent potassium hydroxide per gram. After 30 minutes, the solutions were cooled, diluted with water (twice the volume of ethanol used), and extracted with chloroform (4 times the volume of ethanol).

[4] See footnote 4, page 439.
[6] See footnote 6, page 441.

The aqueous layer was acidified, and the precipitated acids were taken up in fresh chloroform. After the usual treatment,[6] this gave fraction F. The original chloroform solution gave a soap fraction (G) by washing repeatedly with water, as long as the water removed the soap. This was isolated from the water by acidification and extraction with chloroform. The true neutral fraction (H) remained in the original chloroform. The only purpose of taking two acid fractions by the solvent partition was to avoid emulsions. Of course, increasing the amount of water in the partition augments F at the expense of G. Fraction F is the active fraction (tables 1 and 2, and flow-sheet 2).

FLOW-SHEET 2.—*Hydrolysis of the crude extract and separation of hydroxy-acids*



[6] See footnote 6, page 441.

Separation of hydroxy-acids.—The acidic fractions (F) were esterified with ethereal diazomethane. The ester was dried thoroughly by distilling chloroform from it. Treatment with succinic anhydride in dry pyridine gave carbinol succinates and noncarbinols, by the usual partition between ether and 5 percent potassium carbonate solution (*1*). The ratio of product weights was 4:1 in favor of the noncarbinols. The carbinol succinates were hydrolyzed to the hydroxy-acids by heating with alcoholic potash, and these were isolated by acidification with dilute hydrochloric acid and extraction with chloroform.

Treatment of the myeloid agent with Girard reagent.—This was applied to esters of fraction F and also to noncarbinol esters as indicated in table 1. The procedure of Reichstein and von Euw (*5*) was followed, except that the nonketonic material was removed with chloroform and a single ketonic fraction was taken by acidifying to congo paper with 4 N sulfuric acid to decompose the ketonic Girard complex. In table 1 and in table 2, the results of a modified Girard separation are also indicated. In this method, two nonketonic fractions were taken, one soluble in *n*-heptane, and residual nonketones soluble in chloroform but not in heptane.[7] A sample of 47 mg. of esters of fraction F from myeloid leukemia was reacted with 160 mg. of Girard reagent T in 10 ml. of methanol by refluxing gently for 2 hours. After standing at room temperature for 10 hours, the methanol was removed *in vacuo* below 20° C. and the residue was chilled and treated with 10 ml. of ice water. The mixture was rapidly extracted with two 10-ml. portions of cold heptane, and then with five 10-ml. portions of cold chloroform. Both nonketonic fractions were washed with 10 percent hydrochloric acid and then with water, and dried. The aqueous ketonic fraction was treated with 10 ml. of 4 N sulfuric acid, and the liberated free ketones were taken up in chloroform after standing for 10 hours at room temperature. The activity appeared in the fraction of nonketones not soluble in heptane, as shown in table 1. The insolubility of methyl myelokentrate in *n*-heptane made it possible to reduce the active dose to 10 μg. Various dry ester fractions (table 1) were extracted three times with 2 ml. of *n*-heptane at room temperature.

Interconversion of Active Fractions

1) *Reduction of the myeloid agent to material having lymphoid activity.*—A sample (2 mg.) of noncarbinol esters from monocytic leukemia, testing myeloid, was shaken with hydrogen and 2 mg. of Adams' platinum oxide catalyst in 50 ml. of ether for 15 minutes. After isolation, the product was found to be ++L at the same dose (0.6 mg.).

A reduction with sodium borohydride (7) at room temperature was incomplete, the test of ++H indicating a partial reduction to produce a mixture of myeloid and lymphoid agents.

[7] In the Girard separations, myelokentric acid appears in the nonketonic fraction, although it is thought to be a ketone. The modified method was suggested by the discovery of Wintersteiner, Moore, Fried, and Iselin (*6*) that Girard complexes can be soluble in ether and chloroform and thus appear in the nonketonic fraction. It is unlikely that they would ever be soluble in the nonpolar solvent, *n*-heptane. In the case of myelokentric acid, it is evident (tables 1 and 2) that there is little reaction with the Girard reagent.

2) *Oxidation of the lymphoid agent to material having myeloid activity.*—Esters of hydroxy-acids testing ++L, from the plasma of patients with lymphoid leukemia, were oxidized with the pyridine-chromium trioxide complex, using the procedure of Sarett's group (*8*). The product tested myeloid at the same dose (0.2 mg.) but the activity was less. Various other oxidation procedures were tested and resulted in destruction of activity (permanganate), or incomplete oxidation indicated by the test result of +H [oxidation with N-bromoacetamide according to the procedure of Sarett (*9*)].

Chromatographic separation of esters from plasma of patients having monocytic leukemia.—The methyl ester of fraction F (60 mg.) was dissolved in 10 ml. of chloroform and put on a column of 25 gm. of activated alumina (acid washed, Brockmann activity II). Material easily eluted by 100 ml. of peroxide-free ether gave a residue (20 mg.) having activity of +M (100 μg.). A second elution with 100 ml. of ether containing 10 percent acetic acid gave 10.5 mg., with activity of +H (50 μg.). Finally the column was stripped with ether and acetic acid, giving 22 mg. with activity of +L (112 μg.).

The two most adsorbed fractions from the preceding chromatograph were combined, and separated into carbinols and noncarbinols by the established method (table 1). A sample of the hydroxy-acids obtained by hydrolysis of the succinate was separated by the paper chromatographic method of Burton, Zaffaroni, and Keutmann (*10*). By this method, material was obtained that was +L at doses of 20 μg. This activity traveled practically with the solvent front.

Discussion

The chemical separation employed here duplicates our earlier work with urine (*1*). The serum and plasma extracts have the great advantage of being easily purified so that biological effects can be observed with doses in the microgram range.

It seems probable that the substances here are the same as the myelokentric and lymphokentric acid of urine. Both are again shown to occur together in monocytic leukemia; separation can be achieved either by chromatography or by the use of succinate half esters of the lymphokentric acid.

Although Foster and Miller (*4*) obtained negative results when they injected the serum of normal persons, both substances were demonstrated in one sample of normal plasma by the use of doses increased tenfold. This is in accord with the occurrence of both substances in normal organs (*2*, *11*).

The lymphokentric acid is again shown to be a hydroxy-acid by the use of the succinate separation. This is an effective method for purifying lymphokentric acid but not myelokentric acid, which remains with the bulk of the impurities in the noncarbinol fraction. Myelokentric acid has again been reduced to material biologically the same as lymphokentric

acid. Similarly, lymphokentric acid has been oxidized to material producing the myeloid biological reaction.

In order to purify myelokentric acid to have biological activity at doses in the microgram range, it is necessary to depend on solubility or chromatographic separations. Separations resting on chemical functional groups, like the succinate separation, are more trustworthy because of the varying influence of impurities on the physical properties of the fractions. Although the oxidation-reduction experiments show that myelokentric acid is a keto-acid, it fails to react with Girard reagent (*1*).

Summary

1) The biological effects described by Foster and Miller (*4*), produced by the serum of patients with leukemia, have been shown to depend on the presence of two chemical substances in the serum.

2) These substances have been extensively concentrated and have been shown to resemble urinary myelokentric and lymphokentric acids in chemical and biologic properties. The concentration method used for plasma and serum achieves a more effective separation of the agents from impurities, and the doses of material found to produce activity are readily reduced to the microgram range.

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Growth and Survival of Sarcoma 37 in Various Mouse Stocks[1]

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For several years this Laboratory has been screening chemical agents for their ability to produce damage in Sarcoma 37 (*1*, *2*). This is a well-known tumor that has been carried in many laboratories for almost half a century. Originally a spontaneous mammary adenocarcinoma, it underwent transformation into a sarcoma after repeated transplantation. The genealogy of the origin and transformation of Sarcoma 37 was described in detail by Haaland (*3*) in 1908. A histologic description of the tumor was given by Bashford (*4*) in 1911.

In our experiments, CAF_1 hybrids [(BALB/c $\times$ A)F_1] were routinely employed for carrying the tumor. It grew rapidly and progressively in this hybrid, with little evidence of spontaneous necrosis until the later stages of growth, when ulceration became prominent. Until the eighth or ninth day of tumor growth, when our experiments were completed, only minimal necrosis was usually observed.

Due to shortages of CAF_1 mice, it occasionally became necessary to use other strains or stocks of mice in which to carry Sarcoma 37. In such instances, we determined whether the tumor regressed in an appreciable number of mice of the available stocks. In accord with the observations of Andervont (*5*, *6*) we found that Sarcoma 37 grew progressively in some stocks but regressed in others, even though the implants grew in virtually 100 percent of the mice.

These data are presented to re-emphasize the fact that the lack of strain specificity of Sarcoma 37 does not imply that it will grow progressively in any strain or stock with a minimal regression rate.

Materials and Methods

Sarcoma 37, originally obtained from the Crocker Institute, has been carried in our Laboratory since 1932. In recent years, transfer of the tumors has generally been done in CAF_1 hybrids, but in previous years it had been maintained in various strains such as DBA/2, A, C (BALB/c),

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

etc. For about 6 years, the tumor implants have been made intramuscularly; earlier they had usually been made subcutaneously.

Growth of Sarcoma 37 was obtained in the following strains or stocks: C57BL, C3H, DBA/2, A, C (BALB/c), CAF_5, ABC (Bittner),[3] ZBC (Bittner),[3] CF (Carworth Farms), CFW, NIH-B.S., and NIH-Reg. The CFW mice were offspring of Carworth Farms mice bred at the National Institutes of Health by brother × sister mating since 1947. The NIH-B.S. were progeny of brother × sister matings of Swiss line mice. NIH-Reg. were offspring of selected breeding of Swiss stocks.

The tumors were transplanted by inoculation of tissue mash or of small fragments. Precautions were taken to keep the tissue sterile, and the tissue used for transplantation was routinely cultured in a thioglycollate medium for evidence of bacterial contamination.

In many instances, particularly with CAF_1, ABC, and ZBC mice, observations were made over many transplant generations. Generally, at 6 or 7 days after implantation those tumors in a transplant generation that were not used in the screening experiments or for the next transplant generation were set aside and observed for regressions and for survival time. Mice in which tumors had regressed were kept a few weeks and then were killed. In a few instances, tumor measurements were made with calipers and the approximate volumes calculated.

Results

Table 1 summarizes the observations on the growth and regression of Sarcoma 37 in about 2,000 mice of 12 different strains and stocks. Inbred strains are listed first, followed by F_1 hybrids and backcrosses, and finally by stocks of less defined pedigree. The tumor grew progressively without

TABLE 1.—*Regression of Sarcoma 37 in various mouse stocks*

Strain or stock	Number observed	Number dead with tumors	Regressions	
			(number)	*(percent)*
A/L	47	29	18	*38*
A/He	53	52	1	*2*
BALB/c	51	51	0	*0*
C57BL	23	5	18	*78*
C3H	83	25	58	*70*
DBA/2	43	43	0	*0*
CAF_1	559	556	*3	*<1*
ABC	232	232	0	*0*
ZBC	141	141	0	*0*
CF	242	79	163	*67*
CFW	200	109	91	*46*
NIH-B.S.	124	72	52	*42*
NIH-Reg	119	58	61	*51*

* It is not known whether the implants in these three failed to take or whether they regressed.

[3] ABC = backcross $(A \times C3H)F_1$ to A.
ZBC = backcross $(A \times C3H)F_1$ to C3H.

regression in all the BALB/c, DBA/2, ABC, ZBC, and CAF_1 mice (>99 percent). Only 3 of the 559 CAF_1 mice observed were without tumors; it is not known whether the implants did not take in these three mice or whether they took and regressed.

Regression rates of over 40 percent (42 to 78) were observed in C3H, C57BL, CF, CFW, NIH-B.S., and NIH-Reg. In these animals the tumors grew for a period of a week or 10 days and then regressed in a high proportion of the mice.

A smaller percentage of regression (19 percent) was observed with strain A mice. Two sublines of this strain were used, the Heston and Lilly sublines. Only one regression in 53 mice (2 percent) occurred in the Heston subline whereas, in the Lilly subline, the tumor regressed in 18 of 47 mice (38 percent).

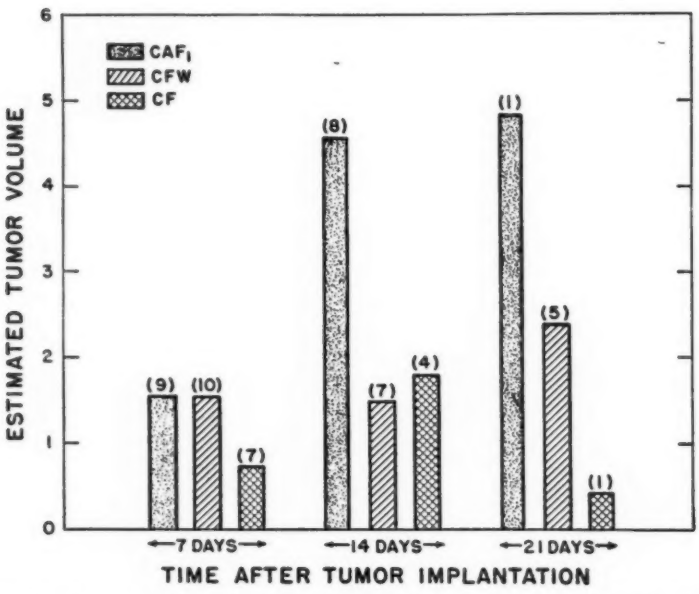
Table 2 lists the average survival time (two to four weeks) of the stocks in which no significant regression occurred. Some of these animals (particularly CAF_1, ABC, and ZBC) were left over from groups selected for use in screening tests, or for transfer of the tumors for the next generation. Usually the most uniform tumors were selected for the latter purpose, so that the survival data were reported for a less uniform population than one would ordinarily get with a random group. It is possible that such selection might have had some influence on the average survival time and on the standard deviation.

TABLE 2.—*Mean survival time of mice bearing Sarcoma 37 in stocks yielding progressive growth of the tumors*

Strain	Number of mice	Mean survival time
		days±*S. D.**
CAF_1	559	20.4 ±5.1
ZBC	141	22.6 ±7.0
ABC	232	26.6 ±9.0
DBA/2	43	16.0 ±5.1
BALB/c	51	20.1 ±5.3

*Standard Deviation.

In several groups of animals, tumor volume was estimated by determining the average diameters with calipers. Similar results were obtained in all the groups. Text-figure 1 illustrates the results obtained in one such experiment; tumor volumes were estimated at 7, 14, and 21 days after intramuscular implantation of the same tumor mash into 10 mice each of CAF_1, CF, and CFW stocks. At first the tumor grew in all the animals in each of the groups, but later it regressed in some of the CF and CFW mice. The estimated volumes include only those surviving mice that had measurable tumors. At 7 days not all the mice had as yet developed measurable tumors. By 14 days 2 CAF_1 mice had died, whereas regressions had already occurred in some of the CF and CFW mice. At 21 days, the relationships were similar to those at the 14-day observation period, except that 9 CAF_1 mice were dead with tumors, and regressions had occurred in additional mice of the other two stocks.



TEXT-FIGURE 1.—Approximate volume of Sarcoma 37 (expressed in cc.) following implantation into CAF_1, CFW, and CF mice. Numbers in parenthesis represent number of mice measured.

Growth of the tumor appeared to proceed at a similar rate in the CAF_1 and CFW mice during the first week. By the second week the progressive growth in CAF_1 mice, coupled with regression in some mice of the other stocks, produced a much greater disparity in the average size of the tumors in the CAF_1, as compared with CF and CFW mice.

Discussion

During recent years there has been increasing interest in the immunogenetics of tumor transplantation (*7–10*) and in the relationship of the chromosomal ploidy of tumors to their heterotransplantability (*7, 11*). Recent symposia at the 1952 Annual Meeting of the American Association for Cancer Research (*8, 9*) and the Tissue Transplantation Conference [4] have emphasized the complexities in transplantability of tumors once the tumor has been carried in other than the inbred line in which it originated (F_1, backcross, hybrids containing the same H-factors as the tumor, etc.). As far as tumors such as Sarcoma 37 are concerned, the picture would appear to be even more complex; the tumor originated in a noninbred animal and has been carried for almost 50 years in a wide variety of hosts, both noninbred and inbred. In addition, the data of Andervont (*5*) indicate that the site of implantation plays a significant role in the ability of Sarcoma 37 to grow progressively in some strains.

[4] Proceedings of the Tissue Transplantation Conference (held at Arden House, Harriman, N. Y., October and 8, 1952). J. Nat. Cancer Inst.: In press.

Despite these factors our own data, collected about 15 years later than Andervont's, show that insofar as the strains examined by both of us [A, BALB/c (C), C3H, DBA/2 (D), C57BL] are concerned, any deviations that occurred both in the strains themselves, or in the immunogenetic adaptation of the tumor, had no significant effect on the progressive growth or regression in these 5 strains. The only exception, possibly, could be strain A. With the small number of animals we have examined, the Lilly subline appears to have deviated from the Heston subline. This would seem to be evident from the difficulties we and others at the National Cancer Institute have had in obtaining successful "takes" in the Lilly subline of Lymphoma 2 (*12*), which originated in the Heston subline.

Walsh *et al.* (*13*) reported 96 percent "takes" of Sarcoma 37 with CAF_1 and Rockland mice, 100 percent with CFW, and 83 percent with St. L.U. stock mice. They did not state explicitly whether these animals were observed for regressions of the tumor, although by implication it appeared that none did regress in the control groups. The origin of the stocks, except for St. L.U., was not given. Neither is it known whether the CFW mice used by them are similar to those we employed, in which 46 percent regression was observed. Furthermore, the latent period before palpable tumors were observed by them (10–15 days) is considerably longer than that commonly experienced by others (5 days). It is possible that their method of homogenization might have disrupted an excessively large number of cells.

Diller *et al.* (*14*) reported about 27 percent regressions of Sarcoma 37 in female Carworth Farms mice and about 14 percent in males of the same stock. Whether this stock is the same as the CF mice we obtained from the same source, in which over 60 percent regressions were observed, is not known.

Hauschka and Levan (*7*) reported no regressions of Sarcoma 37 in the C57BL and C3H lines and in other inbred strains in which the tumor was implanted intraperitoneally in the ascites form. We observed 70 percent or more regressions in C57BL or in C3H. Whether the site or form of the tumor (solid or ascites) are factors in this difference is not established as yet.

Quastel and Cantero (*15*) reported only "very rare" regressions of Sarcoma 37 in CFW mice, but no figures are given for either the number of takes or for the number of regressions. It is noted that, in their experiments, the rate of tumor growth appeared to be much less than we have observed in any host in which Sarcoma 37 grew progressively. In addition, survival time appears to be considerably greater in their experiments. In any case, there is no assurance that their stock was similar to ours.

Despite the fact that Sarcoma 37, as well as other tumors of similar origin, are tetraploid (*11*) and consequently have a less rigid strain specificity than diploid tumors, the point made by Hauschka and Levan (*7*) that these tumors may not be "non-antigenic" in their compatibility to *all* host strains needs to be further emphasized. Our own experience with Sarcoma 37 suggests that progressive growth of this tumor in any strain or stock of mice is by no means as general as is sometimes assumed.

Summary

1) Intramuscular implants of Sarcoma 37 grew progressively in DBA/2 BALB/c, CAF_1, ABC, and ZBC mice.

2) Regression was observed in C3H, C57BL, CF, CFW, NIH-Reg., and NIH-B.S. mice, in percentages ranging from 42 to 78 percent.

3) One regression in 53 mice of the Heston subline of strain A was observed; regression was observed in 18 of 47 mice of the Lilly subline of strain A.

4) Typical growth rates of Sarcoma 37 in different stocks with and without regressions are given.

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ANNOUNCEMENTS

New *Excerpta Medica* Section on Cancer

Excerpta Medica announces publication of a new section on cancer. In this section together with the other 15 are published pertinent and reliable abstracts in English from every available medical journal in the world covering clinical and experimental medicine.

The new Cancer section, like the existing 15 sections of *Excerpta Medica*, will be published monthly. It will contain 700 to 800 pages of informative abstracts a year, including a monthly index of authors and a classified subject and author index at the completion of each yearly volume. The subscription rate for volume I, which will consist of 6 monthly issues from July to December 1953, is $5 (or the equivalent in local currency). Volume II (1954) and succeeding volumes, which will consist of 12 monthly issues, will be priced at $10 a year.

Grants for Scholars in Cancer Research

Applications for Grants for Scholars in Cancer Research are being accepted by the Committee on Growth of the National Research Council, acting for the American Cancer Society.

These awards are designed to bridge the gap between the completion of fellowship training and the period when the young scientist has thoroughly demonstrated his competence as an independent investigator. A grant of $18,000, payable over three years, will be made to each Scholar's institution as a contribution toward his support, his research, or both. Each institution may submit more than one application. These grants are not restricted to the support of individuals who have held American Cancer Society Fellowships. Applications should be submitted by institutions on behalf of a candidate prior to January 1, 1954.

Additional information may be obtained from the Executive Secretary, Committee on Growth, National Research Council, 2101 Constitution Avenue, N. W., Washington 25, D. C.

Medical Research Fellowships

The Division of Medical Sciences of the National Research Council is accepting applications for postdoctoral research fellowships for 1954–1955. These awards are designed to offer research experience for promising individuals who look forward to investigative careers, and not to provide practical experience in the clinical field. Ordinarily Fellowships are not granted to persons over 35 years of age. The following programs are announced:

Fellowships in Cancer Research are awarded by the American Cancer Society on recommendation of the Committee on Growth of the Division of Medical Sciences. Awards are available for study in all branches of the biological, chemical and physical sciences and of clinical investigation applicable to the study of growth, typical or malignant. Citizens of the United States are eligible.

British-American Exchange Fellowships in Cancer Research also are awarded by the American Cancer Society upon recommendation by the Committee on Growth. They are offered to citizens of the United States for advanced study in Great Britain in specialized fields pertaining to the problem of cancer. Similar fellowships are awarded by the British Empire Cancer Campaign to young British scientists for research in the United States.

Fellowships in the Medical Sciences, supported by The Rockefeller Foundation and by The Lilly Research Laboratories, are administered by the Medical Fellowship Board of the Division. Fellows are expected to devote themselves to research in the basic medical sciences. The Rockefeller Fellowships are open to citizens of the United States and Canada; the Lilly Fellowships only to citizens of the United States.

Fellowships in Tuberculosis are also administered by the Medical Fellowship Board under a grant from the National Tuberculosis Association. These awards are designed to promote the development of investigators in fields related to tuberculosis. They are open to citizens of the United States who are graduates of American schools.

Fellowships in Radiological Research are administered by the Division's Committee on Radiology for the James Picker Foundation. The Foundation has expressed particular interest in the support of candidates who propose to carry on research oriented toward the diagnostic aspects of radiology. Appointments are not limited to citizens of the United States.

Applications for 1954–1955 under any of these programs must be postmarked on or before *10 December 1953*. Fellowships are awarded in the late winter or early spring. Complete details and application blanks may be obtained from the *Fellowship Office, National Research Council, 2101 Constitution Avenue, N. W., Washington 25, D. C.*

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The Journal of the National Cancer Institute is published bimonthly by the National Cancer Institute of the National Institutes of Health, Bethesda, Md. Six issues, beginning with number 1 in August, comprise each yearly volume. The Journal publishes original observations and investigations in laboratory and clinical research in cancer. Manuscripts are considered for publication with the understanding that they have not been published previously.

Publication is not limited to members of the National Cancer Institute or to those whose work is supported by Government funds. Manuscripts should be submitted to the Director, National Cancer Institute, Bethesda 14, Md.

Suggestions to Authors

1. Submit an original and two carbon copies of the manuscript. Follow the style of a recent copy of the Journal, especially for illustrations, references, and tables. Type all material double spaced.

2. In the by-line, include the name of the institution with which each author is connected. Official title or academic degree is optional.

3. Begin each page with a new paragraph; *i.e.*, each page should contain one or more complete paragraphs, and no paragraph should carry over from one page to the next unless it is longer than a full page. Do not begin a new paragraph on a page carrying a run-over paragraph. This is a requirement of the Government Printing Office.

4. Insert each footnote in the text immediately after the line in which it is mentioned.

5. Type each table on a separate page, to be the page immediately following the paragraph in which the table is first mentioned.

6. Graphs, charts, and drawings are printed in the text and referred to as text-figures. Legends for text-figures should be included in the text immediately following the paragraph in which the text-figure is first mentioned. The original drawings reproduce best and are therefore preferable. If two additional copies (photostats or etc.) of each drawing are also submitted, review of the manuscript is expedited.

7. Photographs are printed together at the end of each article, as plate pages. The maximum size for each printed plate page is 5¼ by 8 inches, and the photographs should be combined into as few plates as possible. Photographs should be submitted either in the exact size, or exactly double the size, of the final printed reproduction. Mount the photographs for each plate together on one piece of heavy cardboard. Number each photograph, consecutively. Type the legends for each plate on a separate page. If, in addition, two unmounted duplicate prints of each photograph are also submitted, review of the manuscript will be expedited.

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